

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07K 4/02, 14/025, A61K 38/08, 38/10, 38/16, 39/12, 39/165, 39/385</b>		<b>(11) International Publication Number:</b> <b>WO 98/23635</b>
<b>A1</b>		<b>(43) International Publication Date:</b> 4 June 1998 (04.06.98)
<b>(21) International Application Number:</b> PCT/AU97/06820		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent: (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent: (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent: (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent: (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
<b>(22) International Filing Date:</b> 1 December 1997 (01.12.97)		
<b>(30) Priority Data:</b> PO 3903 29 November 1996 (29.11.96) AU		
<b>(71) Applicants (for all designated States except US):</b> THE UNIVERSITY OF QUEENSLAND [AU/AU]; St. Lucia, QLD 4072 (AU). CSL LIMITED [AU/AU]; 45 Poplar Road, Parkville, VIC 3052 (AU).		
<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> FRAZER, Ian, Hector [AU/AU]; 64 Eighth Avenue, St. Lucia, QLD 4067 (AU). TINDLE, Robert [AU/AU]; 14 Cranwood Street, Kenmore, QLD 4069 (AU). AZOURY-ZIADEH, Rania [AU/AU]; 15 Ponsonby Street, Annerley, QLD 4103 (AU).		
<b>(74) Agent:</b> KELLY, Robin, Thomas; Fisher Adams Kelly, Level 13, 10 Eagle Street, G.P.O. Box 1413, Brisbane, QLD 4000 (AU).		<b>Published</b> With international search report.
<b>(54) Title:</b> NOVEL PROMISCUOUS T HELPER CELL EPITOPES		
<b>(57) Abstract</b> <p>The invention is directed to a peptide encoding a promiscuous T helper cell epitope for generating an immune response against papillomavirus, wherein the peptide is selected from the group consisting of (i) VYRDGNPYA inclusive of a single amino acid deletion, substitution or addition made therein (SEQ ID NO. 1); and (ii) QYNKPLCDLL inclusive of a single amino acid deletion, substitution or addition made therein (SEQ ID NO. 2). The invention is also concerned with chimeric constructs as well as immunogenic compositions comprising the peptides of the invention.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TITLE

"NOVEL PROMISCUOUS T HELPER CELL EPITOPES"

FIELD OF INVENTION

5 This invention relates to promiscuous T helper cell epitopes, and in particular, novel promiscuous T helper cell epitopes which have utility in the development of novel peptide-based vaccines. The invention also extends to vaccine compositions which include the novel promiscuous epitopes of the invention which may be useful in eliciting an immune response against PV and particularly HPV (human papillomavirus) in a host  
10 animal.

BACKGROUND OF THE INVENTION

Papillomaviruses induce benign hyperproliferative lesions in humans and in many animal species, some of which undergo malignant conversion. The biology of papillomavirus infection is summarised in a  
15 review by J. P. Sundberg entitled "Papillomavirus Infections in Animals" *In* "Papillomaviruses and Human Disease" edited by K. Syrjanen, L. Gissmann and L. G. Koss, Springer Verlag (1987).

Papillomaviruses are a family of small DNA viruses encoding up to eight early (E1, E2, E3, E4, E5, E6, E7 and E8) and two late genes (L1  
20 and L2). These viruses have been classified in several distinct groups such as HPV which are differentiated into types 1 to 70 depending upon DNA sequence homology. A clinicopathological grouping of HPV and the malignant potential of the lesions with which they are most frequently associated are summarised in "Papillomaviruses and Human Cancer" by H.  
25 Pfister, CRC Press, Inc. (1990). For example, HPV type 1 (HPV-1) is present in plantar warts, HPV-6 or HPV-11 are associated with condylomata acuminata (anogenital warts), and HPV-16 or HPV-18 are common in pre-malignant and malignant lesions of the cervical squamous epithelium.

The immunological approach to the prevention of HPV disease  
30 requires a thorough analysis of the viral proteins against which humoral and cellular immune responses are mounted during and after infection. However,

despite recent limited success (Kreider *et al.*, 1986, *J. Virol.* **59** 369; Sterling *et al.*, 1990, *J. Virol.* **64** 6305; Meyers *et al.*, 1992, *Science* **257** 971; Dollard *et al.*, 1992, *Genes and Development* **6** 1131), papillomaviruses are notoriously refractory to growth in cultured cells (Teichman and LaPorta, 1987 *In "The Papovaviridae"*, Vol. 2 edited by N. P. Salzman and P. M. Howley p. 109) and this has resulted in a lack of viral reagents which has delayed the analysis of the immune response to PV infection.

The recent advent of recombinant expression systems *in vitro* has allowed the production of viral proteins encoded by both early and late genes in relatively large amounts and in a purified form (Tindle *et al.*, 1990, *J. Gen. Virol.* **71** 1347; Jarrett *et al.*, 1991, *Virology* **184** 33; Ghim *et al.*, 1992, *Virology* **190** 548; Stacey *et al.*, 1992, *J. Gen. Virol.* **73** 2337). These systems have, for the first time, allowed the analysis of the host immune response to these viral proteins.

Interest in immune responses to the non-structural early open reading frame (ORF) proteins of HPV has centred on HPV-16 E7 because of an apparent association between serum antibodies to this protein and cervical cancer (for a review, see "Immune Response to Human Papillomaviruses and the Prospects of Human Papillomavirus-Specific Immunisation" by Tindle and Frazer *In "Human Pathogenic Papillomaviruses"* edited by H. zur Hausen, Current Topics in Microbiology Immunology **186**, Springer-Verlag, Berlin, 1994).

The immune responses to other HPV early ORF proteins have also been investigated including HPV-16 E6 (Stacey *et al.*, 1992, *J. Gen. Virol.* **73** 2337; Bleul *et al.*, 1991, *J. Clin. Microbiol.* **29** 1579; Dillner, 1990, *Int. J. Cancer* **46** 703; and Müller *et al.*, 1992, *Virology* **187** 508), HPV-16 E2 (Dillner *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* **86** 3838; Dillner, 1990, *supra*; Lehtinen *et al.*, 1992, *J. Med. Virol.* **37** 180; Mann *et al.*, 1990, *Cancer Res* **50** 7815; and Jenison *et al.*, 1990, *J. Infect. Dis.* **162** 60) and HPV-16 E4 (Köchel *et al.*, 1991, *Int. J. Cancer* **48** 682; Jochmus-Kudielka *et al.*, 1989, *JNCI* **81** 1698; and Barber *et al.*, 1992, *Cancer Immunol. Immunother* **35** 33).

However, comparison of these studies reveals a lack of correlation between the results of the various assays which have been used in assessing HPV early ORF protein reactivity in serum (Tindle and Frazer, 1994, *supra*).

5 In addition, antibodies to other HPV early ORF proteins have not yet been sought with sufficient rigour in large enough numbers of patients to determine their utility as disease markers or as indicators of HPV protein immunogenicity following HPV infection.

10 For many pathogens including papillomaviruses, a conventional killed or live attenuated vaccine cannot be developed because material is not available, or because the vaccine would be unsafe. However, in many cases, immunogenic B and T epitopes (usually between 4 and 20 amino acids in length) have been defined for proteins encoded by these pathogens and this has increased interest in the formulation of peptide-based vaccines.

15 Peptides can be synthesised in large quantities with high purity and are chemically well defined. Synthetic peptides can be designed to incorporate any antigenic B and T epitope (Tindle *et al.*, 1991, *Proc. Natl. Acad. Sci. USA* **88** 5887-5891), and exclude potentially deleterious or dangerous functional domains of a protein (Berzofsky, J. A., 1991, *FASEB J.* **5** 2412-2418). In order to synthesize a highly immunogenic peptide, it is generally considered that uncoupled, carrier free peptide or peptides must contain domains which activate T-helper (Th) cells as well as B-cells so as to facilitate their cognate interaction leading to the development of an effective immune response (Mitchinson, 1971, *Eur. J. Immunol.* **1** 10-17; 20 Mitchinson, 1971, *Eur. J. Immunol.* **1** 18-24; Abbas *et al.*, 1985, *J. Immunol.* **135** 1661-1667). In addition, it has recently been shown that cytokines secreted by Th cells are required for cytotoxic T lymphocyte (CTL) activation which activation is important for effective immune control of tumours and viral infections (Matloubian *et al.*, 1994, *J. Virol.* **68** 8056-8063; Batteggay *et al.*, 25 1994, *J. Virol.* **68** 4700-4704). To this extent, recent experiments have shown that induction of antigen-specific CTL can be effected by linking a Th- 30

cell epitope directly or indirectly to a CTL epitope.

The identification of defined Th-cell determinants and their use to provide effective carrier help to short constructs representing a B-cell epitope or CTL epitope have made it possible to synthesize putative immunogens. However, the use of Th-cell epitopes in the development of potentially immunogenic chimeric constructs has generally been restricted because of the propensity of Th-cells only to provide 'help' to B-cells and/or CTLs displaying the same processed MHC-restricted form of the antigen. Accordingly, the inclusion of Th-cell epitopes in chimeric constructs comprising B-cell and/or CTL determinants is genetically restricted to only one or a few alleles of the MHC with limited activity across divergent MHC class II haplotypes. Consequently, such chimeric constructs have been considered of limited practical value as vaccines targeted to a majority of an outbred population.

Recently, however, universal or promiscuous Th-cell epitopes which can be recognized in the context of several MHC (as well as HLA) haplotypes have been identified in a number of proteins. In particular, Partidos *et al.* (1991, *J. Gen. Virol.* **72** 1293-1299) have identified a Th-cell epitope from the fusion protein (F) of measles virus which is immunogenic in a panel of mouse strains of different H-2 types and in seven of 10 humans tested. Reference also may be made to articles by Panina-Bordignon *et al.* (1989, *Eur. J. Immunol.* **19** 2237-2242) and Ho *et al.* (1990, *Eur. J. Immunol.* **20** 483) which are directed to promiscuous Th-cell epitopes from tetanus toxin that were shown to be immunogenic across a wide spectrum of isotypic and allotypic forms of human MHC molecules. In addition, reference may be made to Sinigaglia *et al.* (1988, *Eur. J. Immunol.* **18** 633-636) which discloses a promiscuous Th-cell epitope from the circumsporozoite protein of *Plasmodium falciparum* which can function as a helper determinant in a wide range of mouse strains and is recognized by most human class II MHC molecules. Regard may also be had to Fridkis-Hareli *et al.* (1994, *Proc. Natl. Acad. Sci. USA* **91** 4872-4876) in which Copolymer 1 (Cop 1), a synthetic

basic random copolymer of amino acids that has been shown to be effective in suppression of experimental allergic encephalomyelitis (EAE), was shown to bind several antigen presenting cells of mouse and human origin irrespective of their MHC class II restrictions.

5                   Moreover, several reports have indicated that peptides representing T- and B-cell determinants constituting a chimeric construct do not have to be derived from the same protein or organism. Indeed, Th-cell epitopes derived from tetanus toxin, sperm-whale myoglobin and streptococcal protein have been found capable of imparting help to B-cell  
10 determinants taken, respectively, from the human malarial parasite *Plasmodium falciparum* (Etlinger *et al.*, 1990, *Science*, **249** 423-425; Kumar *et al.*, 1992, *J. Immunol.* **148** 1499-1505), foot-and-mouth disease virus (Francis *et al.*, 1987, *Nature* **300** 168-170) and hepatitis B virus (LeClerc *et al.*, 1987, *Eur. J. Immunol.* **17** 269-273).

15                   In view of the above, it is known that promiscuous Th-cell epitopes may be incorporated within synthetic chimeric peptides comprising one or more homologous and/or heterologous B-cell determinants in order to bypass haplotype restricted immune responses and thereby alleviate problems associated with vaccinating a genetically diverse outbred  
20 population.

                  In the case of papillomavirus, reference may be made to International Application Publication No WO 92/10513 in which the present applicants disclosed the promiscuous Th-cell minimal proliferative epitope, DRAHYN<sub>I</sub>, which was derived from human papillomavirus (HPV) E7 protein.  
25 This promiscuous epitope either alone or in combination with one or more B-cell epitopes in chimeric constructs was shown to elicit anti-PV proliferative responses in different murine as well as different human MHC backgrounds.

                  In view of the plethora of Th-cell epitopes defined in the literature which epitopes have been derived from a myriad of different  
30 organisms, only a few have been shown to be reactive on several MHC backgrounds (Kaumaya *et al.*, 1993, *J. Mol. Recognit.* **6** 81-94; Domanico *et*

al., 1993, *Eur. J. Immunol.* **23** 1011-1016; Reece et al., 1994, *J. Immunol. Methods* **171** 241-254).

5 The current invention arises from the unexpected discovery that peptide sequences within HPV-16 E6, which have substantially different structures to the DRAHYNI epitope of HPV-16 E7, are reactive on several MHC class II backgrounds. In addition, such HPV-16 E6 peptides when combined with different B-cell epitopes in chimeric peptide constructs elicit specific antibodies which react with peptides containing the B-cell epitopes. In light of the paucity of promiscuous epitopes reported in the literature, this discovery is surprising because it would not be considered reasonably likely that a small organism such a virus, which consists of only a small number of proteins, will comprise a plurality of promiscuous Th-cell epitopes.

#### OBJECT OF THE INVENTION

15 It is therefore an object of the present invention to provide novel peptides comprising promiscuous Th-cell proliferative epitopes which may be utilized to treat HPV infections and which also may be used to provide immunity to HPV infection across a broad spectrum of MHC backgrounds.

20 It is a further object of the invention to provide novel chimeric peptides comprising promiscuous Th-cell proliferative epitopes in combination with one or more B-cell epitopes and/or one or more cytotoxic T-cell epitopes which chimeric peptides may be utilized to generate immune responses against the B-cell epitope(s) and/or the cytotoxic T-cell epitope(s).

#### SUMMARY OF THE INVENTION

25 Accordingly, in one aspect of the invention, there is provided a peptide encoding a promiscuous T helper cell epitope for generating an immune response against papillomavirus, said peptide selected from the group consisting of:-

- 30 (i) VYRDGNPYA inclusive of a single amino acid deletion, substitution or addition made therein (SEQ ID NO 1); and



- (ii) QYNKPLCDLL inclusive of a single amino acid deletion, substitution or addition made therein (SEQ ID NO 2).

The term "promiscuous T helper cell epitope" as used herein refers to a T helper cell epitope which is reactive with a plurality of different MHC class II haplotypes.

The invention also contemplates 'peptide homologs' of peptides according to SEQ ID NO 1 and SEQ ID NO 2. Thus, the invention also includes within its scope peptides which are functionally similar to those defined in SEQ ID NO 1 and SEQ ID NO 2. For example, one of skill in the art will appreciate that conservative amino acid substitutions can be made in peptides according to SEQ ID NO 1 and SEQ ID NO 2 (parent peptides) and that such substituted peptides will retain the functional characteristics of the parent peptides.

The peptides of the invention may be prepared using any suitable procedure. Preferably, such peptides are synthesized either manually or by using an automated peptide synthesiser.

Peptides according to SEQ ID NO 1 and SEQ ID NO 2 and peptide homologs thereof may be synthesised using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled "*Peptide Synthesis*" by Atherton and Shephard which is included in a publication entitled "*Synthetic Vaccines*" edited by Nicholson and published by Blackwell Scientific Publications.

Alternatively, a peptide in accordance with the invention may be prepared by a procedure including the steps of:

- (a) ligating a nucleotide sequence encoding a peptide according to SEQ ID NO 1, SEQ ID NO 2 or peptide homolog thereof into a suitable expression vector to form an expression construct;
- (b) transfecting the expression construct into a suitable host cell;
- (c) expressing the recombinant peptide; and
- (d) isolating the recombinant peptide.

As used in this specification, an expression construct is a nucleotide sequence comprising a first nucleotide sequence encoding a peptide according to SEQ ID NO 1, SEQ ID NO 2 or peptide homolog thereof wherein said first sequence is operably linked to regulatory nucleotide sequences (such as a promoter and a termination sequence) that will facilitate expression of said first sequence. Both constitutive and inducible promoters may be useful adjuncts for expression of the peptides according to the invention. The expression construct preferably includes a vector, such as a plasmid cloning vector. A vector according to the invention may be a prokaryotic or a eukaryotic expression vector, which are well known to those of skill in the art.

Suitable host cells for expression may be prokaryotic or eukaryotic. One preferred host cell for expression of a peptide according to the invention is a bacterium. The bacterium used may be *Escherichia coli*.

The recombinant peptide may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook *et al.* (1989, second edition, Cold Spring Harbor Laboratory Press, in particular Sections 16 and 17).

The term "nucleotide sequence" as used herein designates mRNA, RNA, cRNA, cDNA or DNA.

A nucleotide sequence encoding the peptides of the invention may be conveniently prepared by taking advantage of the genetic code and synthesising, for example, by use of an oligonucleotide sequencer, a sequence of nucleotides which when translated by a host cell results in the production of a peptide according to SEQ ID NO 1, SEQ ID NO 2 or peptide homolog thereof.

It will be understood that each of the peptides according to SEQ ID NO 1 and SEQ ID NO 2 comprises minimal T helper cell proliferative sequences which are considered to incorporate the key "anchor" amino acid residues required for binding an MHC class II molecule. However, it should be noted that peptides associated with MHC class II molecules may

comprise 10 to 34 amino acid residues, and the optimal length of a T helper cell epitope has been defined crystallographically and otherwise to be between 13 and 20 amino acids (Appella *et al.*, 1995, *EXS.* 73 105-119 which is hereby incorporated by reference).

5                   Accordingly, the invention contemplates a peptide of 10 to 34 amino acids corresponding to a natural sequence of amino acids encoded by HPV16 E6 or homologous sequence thereof which natural or homologous sequence comprises the amino acid sequence defined by SEQ ID NO 1 or peptide homolog thereof and/or the amino acid sequence defined by SEQ  
10 ID NO 2 or peptide homolog thereof.

                  In yet another aspect, the invention provides a peptide of 13 to 20 amino acids corresponding to a natural sequence of amino acids encoded by HPV16 E6 or homologous sequence thereof which natural or homologous sequence comprises the amino acid sequence defined by SEQ ID NO 1 or  
15 peptide homolog thereof and/or the amino acid sequence defined by SEQ ID NO 2 or peptide homolog thereof.

                  It will be appreciated from the prior art referred to above and from the exemplification of the invention described hereinafter that such larger peptides will also be expected to behave as promiscuous T helper cell  
20 epitopes.

                  Thus, in a further aspect of the invention, there is provided a chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide in accordance with SEQ ID NO 1 or peptide  
25 homolog thereof linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope.

                  According to yet another aspect of the invention, there is provided a chimeric peptide construct for generating an immune response  
30 against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide in accordance with SEQ ID NO 2 or

peptide homolog thereof linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope.

5 Preferably, the chimeric peptide construct comprises between 10 and 34 amino acids. More preferably, the chimeric peptide construct comprises between 10 and 20 amino acids.

B-cell epitopes may be selected from any suitable source including but not necessarily limited to pathogenic organisms such as pathogenic viruses. A suitable pathogenic virus which may be used as a source of B-cell epitopes includes papillomavirus. For example, the B-cell epitopes may comprise HPV16 E7 B-cell epitopes which include QAEPD, IDGP, EYMLD and YMLD. Alternatively, suitable B-cell epitopes that may be selected from HPV 18 E7 epitopes include DEIDGVNHQL and SEENED.

15 CTL epitopes are preferably selected from a tumor or viral source. In the case of a virus, the virus may be a papillomavirus. In such a case, the CTL epitope may be selected from human CTL epitopes in HPV16 E6 and HPV16 E7 (Ressing *et al.* 1995, *J. Immunol.* **154** 5934-5943 which is hereby incorporated by reference) or the murine CTL epitope RAHYNIVTF in HPV16 E7 (Ossevoort *et al.*, 1995, *J. Immunother.* **18** 86-94 which is hereby incorporated by reference). Alternatively, the CTL epitope may 20 comprise a measles protein CTL epitope as described in an article by Hsu *et al.* (1996, *Vaccine* **14** 1159-1166 which is hereby incorporated by reference) or an Adenovirus CTL epitope as described by Toes *et al.* (1996, *Proc. Natl. Acad. Sci. U.S.A.* **93** 7855-7860 which is hereby incorporated by 25 reference).

Representative peptides which fall within the scope of the invention include the following:

N1 - A - X - A2 (1)

N2 - X - N3 (2)

N1 - A1 - X - A2 - N4 (3)

N2 - X - A2 (4)

A1 - X - N3 (5)

A1 - X - A2 - N4 (6)

N1 - A1 - N4 - A1 - X - A2 (7)

N1 - A1 - N4 - X - N3 - A2 - N5 (8)

5 N1 - A1 - N4 - A2 - N2 - X - A2 (9)

In the above formulae (1) through (9): X denotes a promiscuous T helper cell epitope comprising peptides selected from SEQ ID NO 1, SEQ ID NO 2 or peptide homologs thereof; N1, N4 and N5 represent B-cell epitope or CTL epitope sequences that may be linked to said promiscuous epitope sequences indirectly through intervening sequences of amino acids that are not B-cell or CTL epitope sequences such as A1 and A2.

In some cases, the B-cell or CTL epitope sequence may be linked directly to the promiscuous T helper epitope sequence and in such a case in a first situation the terminal amino acid of the B-cell or CTL epitope sequence and the first amino acid of said promiscuous epitope sequence may be merged. In other cases, in a second situation the last amino acid of the promiscuous T helper epitope sequence and the first amino acid of the B-cell or CTL epitope sequence may also be merged. In this embodiment therefore N2 represents a B-cell or CTL epitope sequence which refers to the first situation and N3 represents a B-cell or CTL epitope sequence that represents the second situation.

The sequences according to SEQ ID NO 1 and SEQ ID NO 2 have been identified as corresponding to two major T helper cell epitopes in the E6 open reading frame (ORF) of HPV16. SEQ ID NO 1 and SEQ ID NO 2 correspond respectively to amino acids 60-68 and 98-107 of the E6 ORF.

Peptides according to SEQ ID NO 1 and SEQ ID NO 2 are each capable of eliciting strong antibody responses to HPV16 E6 challenge across a wide range of MHC class II backgrounds. In addition, chimeric constructs in accordance with the present invention comprising peptides according to SEQ ID NO 1 and SEQ ID NO 2 may facilitate the production

of antibody to several B-cell epitopes simultaneously as well as several CTL epitopes simultaneously.

The invention also includes within its scope an immunogenic composition comprising one or more of the abovementioned peptides and a suitable adjuvant or delivery vehicle.

Suitable adjuvants may be selected from Freund's Complete Adjuvant, Freund's Incomplete Adjuvant, QuilA and saponins generally. Preferably, the adjuvant is capable of eliciting CD8<sup>+</sup> MHC class I restricted cytotoxic T lymphocytes.

Suitable delivery vehicles in which the peptides of the invention may be delivered into a host animal include, but are not necessarily limited to, liposomes, membranous vehicles and microspheres which are well known to those skilled in the art. Alternatively, the delivery vehicle may be an immunostimulating complex (ISCOM). Such ISCOM may be prepared using any suitable technique. For example, reference may be made to Morein *et al.*, 1984, *supra*; Morein, B., 1988, *Nature* **332** 287-288, and Lövgren and Morein, 1991, *Mol. Immunol.* **28** 285-286 which are hereby incorporated by reference. Suitable methods for incorporation of peptides into ISCOMs are also known and these include coupling peptides to influenza virus envelope glycoproteins which have already been incorporated into ISCOMs (Lövgren *et al.*, 1987, *supra*; Lövgren and Larsson, *supra*), or coupling cysteine-containing peptides to preformed influenza ISCOMs using a heterobifunctional reagent (Larsson *et al.*, 1993, *J. Immunol. Methods* **162** 257-260 which is hereby incorporated by reference). Alternatively, peptides may be incorporated into ISCOMs by copolymerisation with the lipid binding peptide LAP20 (Fernando *et al.*, 1994, *In "Vaccines 94"*, pp 327-331, Cold Spring Harbour Laboratory Press).

A preferred delivery vehicle contemplated by the invention is a chimeric VLP including a modified viral capsid protein having one or more peptides of the invention fused thereto. In one form, the chimeric VLP may comprise the modified viral capsid protein alone. Alternatively, the chimeric

VLP may comprise the modified viral capsid protein in association with one or more other viral capsid proteins which may be required for assembly of the chimeric VLP.

5 The modified viral capsid protein may be prepared from any parent viral capsid protein, including a natural viral capsid protein, which when fused with the one or more peptides results in a modified viral capsid protein which is capable of incorporating into a chimeric VLP. The parent viral capsid protein includes, but is not limited to, a papillomavirus capsid protein or a parvovirus capsid protein. Accordingly, the parent viral capsid  
10 protein may comprise an L1 protein or an L2 protein of a papillomavirus. In the case of parvovirus, the parent viral capsid protein may comprise a VP2 capsid protein.

In view of the above, it is well known that foreign peptides can be incorporated into a viral capsid protein to produce chimeric VLPs which  
15 can be used to present foreign antigens to an immune system. Suitable methodologies for production of such chimeric VLPs are also known. For example, the peptides of the invention may be fused to a papillomavirus 1 L1 capsid protein as described, for example, in Muller *et al* (1997, *Virology* **234** 93-111), or fused to a papillomavirus L2 capsid protein as described, for example, in United States Patent No 5,618,536 (Lowy *et al*) which is hereby  
20 incorporated by reference, or fused to a porcine parvovirus VP2 capsid protein as described, for example, in Sedlik *et al* (1997, *Proc. Natl. Acad. Sci. USA* **94** 7503-7508) which is hereby incorporated by reference.

The above exemplary methods of producing chimeric VLPs  
25 essentially involve construction of a synthetic DNA molecule encoding the modified viral capsid protein and subsequent expression of this protein in a suitable host cell to facilitate assembly of the chimeric VLP. Depending on the modified viral capsid protein being expressed, such expression may require co-expression of one or more other viral capsid proteins for assembly  
30 of the chimeric VLP.

The synthetic DNA molecule may be prepared using any

suitable method for altering DNA. Such methods are well known to those of skill in the art and are described for example in the relevant sections of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Ausubel, *et al.*, eds.) (John Wiley & Sons, Inc. 1995) and of Sambrook, *et al.*, MOLECULAR CLONING. A LABORATORY MANUAL (Cold Spring Harbor Press, 1989) 5 which are hereby incorporated by reference. Alternatively, suitable methods for altering DNA are set forth, for example, in U.S. Patent Nos 4,184,917, 4,321,365 and 4,351,901 which are hereby incorporated by reference.

The synthetic DNA molecule may be ligated into a suitable expression vector to produce a recombinant expression vector may be 10 introduced subsequently into a host cell for expression of the modified viral capsid protein. Preferably, the expression vector is a baculovirus expression vector and the host cell is a *Spodoptera frugiperda* 9 (Sf-9) insect cell.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of example with reference to the following figures in which:

FIG. 1 refers to a set of overlapping peptides (termed GF51-GF66) spanning the putative HPV16 E6 protein molecule;

20 FIG. 2 illustrates the overlapping pattern of the peptides of FIG. 1 relative to the amino acid sequence of HPV16 E6 protein;

FIG. 3A is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with saline: CFA, and challenged with individual HPV16 E6 peptides;

25 FIG. 3B is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with saline: CFA, and challenged with PPD + individual HPV16 E6 peptides;

FIG. 4A is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized with peptides GF51-GF54;

30 FIG. 4B is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized with peptides GF55-GF58;



FIG. 4C is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized with peptides GF59-GF62;

FIG. 4D is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized with peptides GF63-GF66;

5        FIG. 5A illustrates the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with an equimolar mixture of peptide GF56;

FIG. 5B is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with an equimolar mixture of peptide GF57;

FIG. 5C is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with an equimolar mixture of peptide GF61;

FIG. 5D is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with an equimolar mixture of peptide GF66;

FIG. 5E is a bar graph showing the *in vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with an equimolar mixture of peptide GF51;

FIG. 6A is a bar graph showing the T-proliferative response of LNCs from C57BL/6 (H-2<sup>b</sup>) mice immunized with an equimolar mixture of peptides GF56, GF57, and GF61 in CFA, and challenged *in vitro* with individual peptides;

FIG. 6B is a bar graph showing the T-proliferative response of LNCs from B10 (H-2<sup>a</sup>) mice immunized with an equimolar mixture of peptides GF56, GF57, and GF61 in CFA, and challenged *in vitro* with individual peptides;

FIG. 6C is a bar graph showing the T-proliferative response of LNCs from B10 (H-2<sup>k</sup>) mice immunized with an equimolar mixture of peptides GF56, GF57, and GF61 in CFA, and challenged *in vitro* with individual peptides;

FIG. 6D is a bar graph showing the T-proliferative response of LNCs from B10 (H-2<sup>d</sup>) mice immunized with an equimolar mixture of peptides GF56, GF57, and GF61 in CFA, and challenged *in vitro* with individual peptides;

5                   FIG. 6E is a bar graph showing the T-proliferative response of LNCs from B10 (H-2<sup>h2</sup>) mice immunized with an equimolar mixture of peptides GF56, GF57, and GF61 in CFA, and challenged *in vitro* with individual peptides;

10                   FIG. 6F is a bar graph showing the T-proliferative response of LNCs from B10 (H-2<sup>h4</sup>) mice immunized with an equimolar mixture of peptides GF56, GF57, and GF61 in CFA, and challenged *in vitro* with individual peptides;

FIG. 7 illustrates the mapping of the minimal T-cell proliferative epitope in the GF57 peptide of HPV16E6 protein;

15                   FIG. 8A is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF57, and challenged with 20 µg/mL GF57 overlapping truncated peptides;

20                   FIG. 8B is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF57, and challenged with 5 µg/mL GF57 overlapping truncated peptides;

FIG. 9 illustrates the mapping of the minimal T-cell proliferative epitope in the GF61 peptide of HPV16E6 protein;

25                   FIG. 10A is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF61, and challenged with 20 µg/mL GF57 overlapping truncated peptides;

30                   FIG. 10B is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF61, and challenged with 5 µg/mL GF57 overlapping truncated

peptides;

FIG. 11A is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF51, and challenged with 20 µg/mL GF57 overlapping truncated peptides;

FIG. 11B is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF51, and challenged with 5 µg/mL GF57 overlapping truncated peptides;

FIG. 12A is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF51, and challenged with 20 µg/mL GF61 overlapping truncated peptides;

FIG. 12B is a bar graph showing the *in vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF51, and challenged with 5 µg/mL GF61 overlapping truncated peptides;

FIG. 13A is a bar graph showing the effect of 'VYRDGNPYA' T<sub>h</sub>-epitope on the antibody response to 'EYMLD' B-epitope;

FIG. 13B is a bar graph showing the effect of 'QYNKPLCDLL' T<sub>h</sub>-epitope on the antibody response to 'EYMLD' B-epitope.

FIG. 13C is a bar graph showing the effect of 'VYRDGNPYA' T<sub>h</sub>-epitope on the antibody response to 'QAEPD' B-epitope;

FIG. 13D is a bar graph showing the effect of 'QYNKPLCDLL' T<sub>h</sub>-epitope on the antibody response to 'QAEPD' B-epitope; and

FIG. 14 is a map of recombinant vector pVLBPV1L1/Thprom-QAEPD.

#### PREFERRED EMBODIMENTS

#### EXAMPLE 1 Identification of T-proliferative epitopes in H-2<sup>b</sup> mice

##### MATERIALS AND METHODS

##### Buffers

Unless otherwise specified, buffers were prepared according to Sambrook *et al.* (1989, MOLECULAR CLONING. A LABORATORY MANUAL, second edition, Cold Spring Harbor Laboratory Press) and Ausubel *et al.* (1995, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, Inc.).

#### Antigens

HPV16 E6 and HPV16 E7 proteins were produced as MS2-fusion proteins from a heat-inducible phage promoter in a pPLc24 expression vector (provided by L. Gissmann, DKFZ, Heidelberg, Germany) in *E. coli* C600/537 by sequential urea extraction, as described by Seedorf *et al.* 1987.

The expression vector pPLc24 allows the expression of inserts fused to the N-terminal part (the first 98 aas) of the MS2 polymerase under the control of the lambda P<sub>L</sub> promoter which does not contain the *cl* gene coding for repressor (Remaut *et al.*, 1981, *Gene* **15** 81-93; Remaut *et al.*, 1983, *Gene* **22** 103-113; Remaut *et al.*, 1983, *Nucleic Acids Res.* **11** 4677-4688). But since the *E. coli* C600/537 strain harbors a temperature-sensitive *cl* repressor gene of phage lambda on a multicopy plasmid conferring kanamycin resistance, expression of the pPLc24 vector was induced in log phase cells by incubating at 42°C for 3 hrs. After induction, cells of a 1 L LB broth culture were first washed with Bacterial Wash Buffer, then lysed in 40 mL Bacterial Lysis Buffer in the presence of 8 mg lysozyme and incubated, with shaking, at 37°C for 30 min. Next, MS2-replicase fusion proteins were partly purified by the addition of 0.1% Triton X-100. After sonicating (on ice) and stirring for 30 min at 37°C, the lysate was cleared by centrifugation at 40,000g for 15 min. The supernatant was removed and stored at -70°C and the pellet was successively extracted with 30 mL Phosphate Buffered Saline (PBS) containing 0.1% Triton X-100, then with 30 mL 3M Urea, and finally with 30 mL 7M Urea. Accompanying all these final 3 steps are sonication, further stirring at 37°C (or at room temperature, RT, in case of urea extraction) and centrifugation; and each time the supernatant was removed

and stored away at -70°C. The final pellet was also stored at -70°C for further testing. After performing SDS-PAGE, Western Blotting and estimating the percentage of purity of the 7M Urea supernatant, the latter was dialyzed against PBS at 4°C, using a 12,000 mwt cut off membrane.

5                    SDS-PAGE / Western Blotting

SDS-PAGE was performed to estimate the purity and yield of the fusion proteins. Consequently, the fusion proteins (~20 µg protein per lane) were diluted in 2x concentrated SDS Sample Buffer containing 0.2 M Dithiothreitol (DTT), heated for 5min at 100°C and separated on a 12% polyacrylamide gel (Laemmli, 1970, *Nature* **227** 680), at 200 V using SDS-PAGE Electrode Running Buffer. This was done in duplicate: one gel was stained with Coomassie Blue, destained with Methanol/Acetic Acid (Destains 1 and 2) and then dried on a slab gel dryer (Drygel Sr. Model SE 1160, Hoefer Scientific Instruments, San Francisco) for 1 hr at 80°C. The other gel was transferred by Western Blotting onto a nitrocellulose membrane using SDS Transfer Buffer at a 100 V according to standard procedures. The blot was blocked with 5% skim milk powder in PBS Blocking Buffer (PBS/milk) at 4°C o/n. The second day, it was incubated at RT for 1-1.5 hr with a rabbit polyclonal anti HPV16 E6/MS2 antiserum (previously prepared in our laboratory) diluted 1:200 in PBS/milk. This was followed by 3 washes (each for 10 min) with the following Wash Buffer: 5% skim milk powder in PBS + 0.1% Triton X-100.

Following washing; a second antibody, Horse radish peroxidase linked sheep anti-mouse immunoglobulin, diluted 1:1000 in PBS/milk was added and the blot was incubated at RT for another 1-1.5hr followed by the washing procedure mentioned above. Finally the specific bands were visualized by the Di-aminobenzidine (DAB) substrate reaction in the presence of H<sub>2</sub>O<sub>2</sub>. The reaction was stopped by rinsing the blot with distilled water (dH<sub>2</sub>O) and air drying.

30                    Protein Concentration Determination

Protein concentration estimation was performed using the

Pierce BCA Protein Assay Reagent commercial kit. The kit contains two reagents: Reagent A (Base reagent) which contains sodium carbonate, sodium bicarbonate, bicinchoninic acid (BCA) detection reagent and sodium tartrate in 0.2 N NaOH; and Reagent B which is a 4% copper sulfate solution.

5 Ten microlitres of sample (or bovine serum albumin, BSA, standard or blank) were mixed with 200  $\mu$ L Working Reagent (50 parts Reagent A + 1 part Reagent B) in microtiter plate wells (usually done in triplicates). After incubating the plate at 37°C for 30 min, absorbance at 540 nm (OD540) was determined by spectrophotometry using a Titertek Multiskan reader.

The protein concentration was estimated by comparison of the mean OD540 (mean of 3 wells) obtained with the sample, with that obtained with the BSA standards.

#### 15 Mice

Generally, inbred mouse strains were obtained from the Animal Resources Centre (Perth, Australia) and were used at 7-10 weeks of age. In regard to inbred Balb/c mice, these were obtained from the same source and were used at 4-12 weeks of age.

#### 20 Synthetic Peptides

Overlapping synthetic peptides (GF51-GF66) covering the entire HPV16 E6 ORF were used as antigens. These peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 431A peptide synthesizer. After obtaining the 'peptide-resin' conjugates, the actual peptide was next cleaved from its resin by the addition of thioanisole and ethanedithiol, and then ether purified.

25 Briefly, 100 mg of resin conjugate, 100  $\mu$ L dH<sub>2</sub>O, 100  $\mu$ L thioanisole and 50  $\mu$ L ethanedithiol were mixed in a glass tube. To this mixture, few phenol crystals dissolved in 2 mL trifluoroacetic acid (TFA) were added and the whole mixture was left to stir at RT for 1.5-2.0 hr. The resin solution was then filtered through a coarse sintered glass funnel, into 40 mL

of cold ether in a 50 mL Falcon tube, and spun down at 4°C, for 5 min, at a speed of 1000g. After discarding the supernatant, the peptide pellet was precipitated 5 more times, each time with 20 mL of cold ether. Finally, the peptide crystals were allowed to air dry o/n and then stored at -70°C.

5                Series of peptides, truncated by 1 aa from either the N-terminal or the C-terminal, and covering the whole length of peptides GF57 and GF61 were synthesized by Chiron Mimotopes Peptide Systems PTY, LTD (Victoria, Australia). The HPLC profile, amino acid composition, toxicity and mitogenicity of all peptides were checked.

10                HPV16 E6 Protein

HPV16 E6 protein was produced as an MS2 fusion protein as described above. Fusion protein was then dialyzed against PBS for use in proliferation assays.

Lymph Node Cell (LNC) Proliferation Assays

15                Mice were immunized subcutaneously in the base of the tail with about 50 µg of peptide emulsified in Complete Freund's Adjuvant (H37 Ra. CFA, Difco). Nine days later, the inguinal and periaortic lymph nodes were collected from the immunized mice, and a suspension of draining LNCs was prepared after two washes in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 100 IU/mL Penicillin, 100 µg/mL Streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES buffer and 50 µM β-mercaptoethanol (complete DMEM, cDMEM).

20                The LNCs suspension was prepared at a concentration of  $2 \times 10^6$  cells per mL in cDMEM containing 10% FCS. A four day proliferation assay (at 37°C) (Good *et al.*, 1987, *Science* **235** 1059-1062) was conducted in peptide-coated microtiter plates where every peptide-coated well was allowed to interact with  $4 \times 10^5$  LNCs/mL (200 µL of LNCs suspension). Wells coated with Purified Protein Derivative (PPD) or cDMEM were used as positive and negative controls, respectively. Next, proliferation was  
25                quantified by [<sup>3</sup>H] Thymidine incorporation: the LNCs were pulsed with 1 µCi  
30                of [<sup>3</sup>H] Thymidine (Amersham) per well; and 16 hr later, the amount of [<sup>3</sup>H]

Thymidine uptake by the cells DNA was determined using an LKB beta-plate system reader.

## RESULTS

### Identification of T-proliferative epitopes in H-2<sup>b</sup> Mice

5 A panel of overlapping E6 16-23 mer peptides covering the whole length of the HPV16 E6 molecule (FIGS. 1, and 2) was tested for lack of toxicity and mitogenicity. For this, lymph-node cells (LNCs) from H-2<sup>b</sup> mice immunized with saline: CFA were challenged *in vitro* with individual HPV16 E6 peptides with or without purified protein derivative (PPD),  
10 respectively. LNC proliferation with PPD was expected since PPD of tuberculin is a component of CFA. According to FIGS. 3A and 3B, none of the peptides seemed to be toxic or mitogenic. Using this panel of non-toxic, non-mitogenic HPV16 E6 peptides, three potential murine T-proliferative epitopes in HPV16 E6 were identified, in T-proliferation assays in which  
15 LNCs from peptide immunized H-2<sup>b</sup> mice were challenged *in vitro* with whole protein and individual peptides.

Initially, four groups of C57BL/6 (H-2<sup>b</sup>) mice were immunized with equimolar mixes of peptides GF51 - GF54, GF55 - GF58, GF59 - GF62 or GF63 - GF66 respectively in CFA. Pooled LNCs from each group were  
20 challenged *in vitro* with individual peptides at 2 or 20 µg/mL.

Peptide GF57 elicited strong proliferation and GF56 weaker proliferation in LNCs from the GF55 - GF58 immunized group (FIGS. 4A, 4B, 4C and 4D). Moreover, a proliferative response was elicited with peptide GF61 in LNCs from the GF59 - GF62 immunized group (FIGS. 4A, 4B, 4C  
25 and 4D). No further peptides from GF51 - GF66 series induced proliferation in assays using LNCs from appropriately immunized H-2<sup>b</sup> mice.

To determine the minimal dose of peptide which would elicit proliferation, C57BL/6 mice were then immunized with mixtures of peptides GF56, GF57, GF61 and irrelevant peptide GF66 in CFA. LNCs from these  
30 mice were challenged *in vitro* with individual peptides at different concentrations (20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL and



0.63  $\mu\text{g/mL}$ ). FIGS. 5A, 5B, 5C, 5D and 5E shows that the LNCs response to peptides GF56, GF57 and GF61 decreased with decreasing concentration of the challenging peptide and that the minimal dose of challenging peptide which would still elicit good proliferation is 5  $\mu\text{g/mL}$ . No response to  
5 challenging control peptides GF66 or GF51 was noticed.

To examine the major histocompatibility complex (MHC) restriction of the response to the immunogenic peptides from HPV16 E6, C57BL/6 mice (H-2<sup>b</sup>) and B10 congenic mice differing only at the major histocompatibility complex (MHC) class 2 locus; B10A (H-2<sup>a</sup>), B10BR (H-2<sup>k</sup>),  
10 B10D2 (H-2<sup>d</sup>), B10A.2R (H-2<sup>h2</sup>) and B10.4R (H-2<sup>h4</sup>) were immunized with mixtures of peptides GF56, GF57 and GF61. LNCs from all strains of immunized B10 congenic mice showed strong proliferative response to peptide GF56. Immunized C57BL/6 mice responded to all three peptides GF56, GF57 and GF61. None of the mice responded to the control peptide  
15 GF51 (FIGS. 6A, 6B, 6C, 6D, 6E and 6F). Hence, peptides GF57 and GF61 each contains a T-proliferative epitope(s) reactive in H-2<sup>b</sup> mice; while peptide GF56 contains a "promiscuous" T-epitope, reactive on several murine MHC backgrounds.

To determine the minimal T-proliferative epitopes sequences, LNCs from GF57 immunized mice were challenged *in vitro* with a series of overlapping peptides, truncated by 1 amino acid from either the N-terminal or the C-terminal, and covering the whole length of peptide GF57 (FIG. 7) at different concentrations (20  $\mu\text{g/mL}$ , 5  $\mu\text{g/mL}$ , 2  $\mu\text{g/mL}$  and 0.5  $\mu\text{g/mL}$ ). LNCs stimulated with peptides GF57/1-3 and GF57/16-24 proliferated significantly,  
25 indicating that the consensus sequence "VYRDGNPYA" was the minimal proliferative epitope (FIGS. 7, 8A and 8B).

Similarly, a series of N-terminal or C-terminal truncated overlapping peptides, covering the whole length of peptide GF61 was used in LNC proliferation assays to recall T-cell activation from mice immunized  
30 with GF61 (FIG. 9). The LNCs proliferated significantly when challenged *in vitro* with peptides GF61/1-3, GF61/14-16 and GF61/19, indicating that the

consensus sequence "QYNKPLCDLL" was the minimal proliferative epitope in peptide GF61 (FIGS. 9, 10A and 10B). It is noteworthy that the LNCs did not proliferate when challenged with peptides GF61/17-18, probably because of the Leucine and Isoleucine being two hydrophobic residues at the C-terminal end of the peptide molecule competing to fit into the T-cell receptor groove on the antigen presenting cell (APC) and resulting in an unstable peptide molecule.

For both of these experiments a control group of mice was immunized with the irrelevant peptide GF51, and LNCs challenged *in vitro* with either the GF57 or the GF61 series of overlapping peptides. However, no response with any of the truncated peptides was noted (FIGS. 11A, 11B, 12A and 12B).

Confirmation of the T-Helper Epitopes in H-2<sup>b</sup> Mice

To determine whether the defined T-proliferative epitopes can provide help for the production of specific antibodies to a characterized B-cell epitope, H-2<sup>b</sup> mice were immunized three times at two weekly intervals with a subunit vaccine consisting of synthetic peptides containing either VYRDGNPYA or QYNKPLCDLL T-epitope linked to known HPV16 E7 B-cell epitopes (Tindle *et al*, 1990, *J. Gen. Virol.* **71** 1347-1354):

EYMLD AG VYRDGNPYA AG QAEPD or  
B T B

EYMLD AG QYNKPLCDLL AG QAEPD  
B T B

wherein AG denotes a neutral spacer between the epitope sequences.

Ten days after the third immunization, sera were then collected from peripheral blood and tested in ELISA assays against peptides containing epitopes QAEPD and EYMLD. These tests showed that after

vaccination with either of these synthetic peptides, the mice developed antibodies only to QAEPD. The absence of anti-EYMLD antibodies in these sera suggests the possibility that the H-2<sup>b</sup> mice have seen the QAEPD as an immunodominant B-epitope and directed most of their humoral immune response to generate antibodies to it. Control mice immunized with either EYMLD alone, QAEPD alone or T-epitope alone did not produce any antibody to either EYMLD or QAEPD (FIGS. 13A, 13B, 13C and 13D).

Hence, the defined proliferative T-epitopes in HPV16 E6 are actually functional T-helper epitopes that can be used for eliciting cognate interaction between T- and B-lymphocytes for the production of antibody against the B-cell epitopes.

#### DISCUSSION

In this study, we have defined three murine T-cell epitopes in the context of H-2<sup>b</sup> haplotype, by observing T-cell proliferation using cells from HPV16 E6 peptide immunised C57BL/6 mice. For this purpose, a set of overlapping peptides (GF51-GF66) spanning the entire HPV16 E6 sequence was used.

The '51DFAFRDLCIVYRDGNPYA<sup>68</sup>' peptide GF56 was shown to contain a promiscuous T-proliferative epitope, reactive on several MHC backgrounds.

Using a series of overlapping, truncated E6 peptides in T-proliferation assays to recall T-cell activation, we were able to define the minimal proliferative sequences of two T-epitopes in HPV16 E6 protein: '60VYRDGNPYA<sup>68</sup>' and '96QYNKPLCDLL<sup>107</sup>'.

When H-2<sup>b</sup> mice were immunized with an equimolar mixture of peptides GF57 and GF61 (FIGS. 6A - 6F), 'VYRDGNPYA' in GF57 seemed to be the dominant epitope since LNC proliferation after *in vitro* challenge with GF57 was stronger than with GF61. T-epitope immunodominance depends on several mechanisms such as antigen processing, competition for binding to MHC, hindering structures outside the minimal epitopes and structures of the T-cell site (Partidos and Steward, 1992, *J. Gen. Virol.* 73

1987-1994; Boehncke *et al.*, 1993, ; Grewal *et al.*, 1995; Moudgil *et al.*, 1996).

Although 'VYRDGNPYA' is a common sequence to both peptides GF56 and GF57, peptide GF57 triggered in H-2<sup>b</sup> mice a stronger proliferative response than GF56. However, the proliferative responses of the other tested strains of mice was stronger with GF56 than with GF57. This finding would suggest that the flanking sequences outside the minimal epitope could influence response as observed by other scientists (Gammon *et al.*, 1987, *Immunological Reviews* **98** 53-73; Partidos and Steward, 1992, *supra*; Brett *et al.*, 1988, *J. Exp. Med.* **168** 357-373; Vacchio *et al.*, 1989, *J. Immunol.* **143** 2814-2819). It is also possible that GF56 contains a T-epitope other than 'VYRDGNPYA', which is proliferative on several MHC haplotypes.

We have also demonstrated that immunisation of mice with either 'VYRDGNPYA' or 'QYNKPLCDLL' joined to B-cell epitopes will elicit specific antibodies which will react with peptides containing the B-cell epitope. This suggests that these two sequences are T-helper-cell-stimulating epitopes that can be used for eliciting cognate interaction between T- and B- lymphocytes for the production of antibody against the B- epitopes.

It is worth mentioning that both 'VYRDGNPYA' and 'QYNKPLCDLL' are located adjacently to 'Cys-X-X-Cys' motifs, 'VYRDGNPYA' being localised to the first loop formed by the four 'Cys-X-X-Cys' motifs present in HPV16 E6 and 'QYNKPLCDLL' present in the region between the loops. It is believed that these motifs play an important role in the function of the E6 protein. They are involved in metal binding and have been shown to be involved in transcriptional regulation (Lamberti *et al.*, 1990, *EMBO J.* **9** 1907-1913) and contribute to E6-mediated transformation by binding to p53 and enhancing its degradation. It appears that the putative p53-binding domain of E6 identified by Crook *et al* (1991, *Cell* **67** 547-556) can be localised to the second loop formed by the four 'Cys-X-X-Cys' motifs. However, the region necessary to direct p53 degradation resides in the first

loop and sequences N-terminal to this. The region between the loops functions as a spacer to keep the loop apart.

In summary, 'VYRDGNPYA' and 'QYNKPLCDLL' are capable of eliciting cognate interaction between T- and B-lymphocytes and therefore may be used generically to produce a subunit synthetic peptide vaccine consisting of a promiscuous T helper cell epitope according to the invention, in combination with one or more B-cell epitopes and/or one or more CTL epitopes, to generate immune responses against the B-cell and/or CTL epitopes. Moreover, the defined promiscuous T-helper epitopes in HPV16 E6 protein are reactive with a plurality of different MHC class II haplotypes and thus may be used in a vaccine construct to treat HPV infections by providing immunity to HPV infection across a broad spectrum of MHC backgrounds.

It is important to note though, that LNCs from H-2<sup>b</sup> mice immunized with HPV16 E6/MS2 protein failed to proliferate when challenged *in vitro* with E6 peptides GF51-GF66. This result may be due to unfolding and antigen processing of the protein molecule. The existence of MHC-specific hindering structures on the naturally processed fragments that are not present on synthetic peptides comprising little more than the antigenic site may interfere with binding to Ia or to the T-cell receptor (TCR) (Brett *et al.*, 1988, *supra*; Partidos and Steward, 1992, *supra*; Sercarz *et al.*, 1993; Moudgil *et al.*, 1996, *supra*). This may have important implications for the design of peptide vaccines that, although immunogenic themselves, may generate a T-cell response that is poorly reactive to the native molecule.

25

**EXAMPLE 2 Preparation of a chimeric VLP comprising VYRDGNPYA and QAEPD fused to the C-terminus of BPV1 L1**

Construction of recombinant baculovirus transfer vector

Primers 5'-CCGCAATCCATGGCGTTGTGGCAACAAGGCC-AGAAGC-3' and 5'-CCGGAATTCTTATTTTTTATCAGGTTTCAGCTT-GAGCATAAGGATTGCCATCCCGATAAACTCCTGCCCCTTGCTGTGCT

30

AAAAATCTTCTTCC-3' may be utilized to allow amplification by PCR of a hybrid nucleotide sequence encoding a modified BPV1 L1 gene in which a chimeric peptide comprising the minimal promiscuous epitope VYRDGNPYA and the HPV16 E7 B-cell epitope QAEPD, is fused to the C-terminus of the BPV1 L1 sequence. This fusion would result in deletion of nucleotides from 7625 downstream of the BPV1 nucleotide sequence. The above primers would also facilitate insertion of a stop codon, and one flanking *Bam*HI site at the 5' end and a flanking *Eco*RI site at the 3' end of the hybrid nucleotide sequence. After amplification, the PCR products could be digested with *Bam*HI and *Eco*RI and inserted subsequently into the baculovirus transfer vector pVL1393 (Pharmingen) at the *Bam*HI and *Eco*RI sites thereof before transformation of DH-5 $\alpha$  cells. Recombinant clones having the correct sequence could be confirmed by restriction endonuclease and nucleotide sequence analyses. A map of a recombinant baculovirus transfer vector pVLBPV1L1/Thprom-QAEPD so produced is shown in FIG. 14.

Production of recombinant baculoviruses

Recombinant baculoviruses could be produced according to Pharmingen's BaculoGold™ transfection kit. Briefly, *Spodoptera frugiperda* 9 (Sf-9) insect cells are co-transfected with BaculoGold™ linearized DNA and the recombinant transfer vectors. Preferably, plaque purification is used to ensure no non-recombinant (wild-type) plaques are detected. High-titer (> 10<sup>8</sup>/mL virus particles) stocks of recombinant baculoviruses are obtained subsequently by two rounds of amplification.

Production and purification of chimeric VLPs

Procedures for production and purification of VLPs have been described in detail previously (Qi *et al.*, 1996, *Virology* **216** 35-45 which is hereby incorporated by reference). In general, Sf-9 insect cells are infected either with recombinant baculovirus at a multiplicity of infection of 10 plaque-forming-units (PFU) per cell, and incubated at 27°C for 72 h. The cells are then centrifuged, washed with PBS once, and resuspended in an appropriate amount of PBS in the presence of 2 mM PMSF. The cell

suspension is then homogenized with a dounce homogenizer (tight pestle) by 50 strokes on ice and then centrifuged at 3000 rpm for 10 min at 4°C to separate the nuclear fraction. The nuclear pellet is then resuspended in an appropriate resuspension buffer and sonicated for 45 sec on ice. The  
5 nuclear suspension is then loaded onto a 20% sucrose cushion and centrifuged at 26,000 rpm in a Beckman SW-26 rotor at 4°C for 2 h. The pellets are then resuspended in resuspension buffer and sonicated again for another 45 sec. The resulting sonicate is then mixed with CsCl and centrifuged at 21°C in a Beckman SW 41 rotor for 20 H. A band at the CsCl  
10 density of about 1.30 g/mL is then collected and dialysed against PBS. The sample may then be used for Western immunoblotting and for transmission electron microscopic analysis for confirming respectively the integrity of the modified BPV1 L1 capsid protein and chimeric VLP structure.

\*\*\*\*\*

15 The present invention has been described in terms of particular embodiments found or proposed by the present inventors to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments  
20 exemplified without departing from the scope of the invention. All such modifications and changes are intended to be included within the scope of the appended claims.

FIGURE LEGENDS

5 **FIG. 1.** A set of overlapping peptides (termed GF51-GF66) spanning the putative HPV16 E6 protein molecule. These peptides were synthesized by Fmoc chemistry. Peptides are shown using the single letter code. The numbers of the first and last amino acids of each peptide correspond to amino acid numbers of the HPV16 E6 polypeptide sequence.

10 **FIG. 2.** Overlapping pattern of the peptides of FIG. 1 relative to the amino acid sequence of HPV16 E6 protein. The first and last amino acid (single letter code) indicated for each peptide correspond to the amino acid numbering of HPV16 E6 protein.

15 **FIGS. 3A and 3B.** Mitogenicity and toxicity assays of the HPV16 E6 overlapping peptides. Each recall peptide was used at two different concentrations: 20 µg/mL (hatched bars), and 2 µg/mL (dotted bars). "NIL" represents background (no added antigen).

20 **FIGS. 4A - 4D.** LNC proliferation assays. Each recall peptide was used at two different concentrations: 20 µg/mL (hatched bars), and 2 µg/mL (dotted bars). "NIL" represents background (no added antigen). Irrelevant peptides GF55 (FIG. 4A), GF59 (FIG. 4B), GF63 (FIG. 4C) and GF51 (FIG. 4D) were used as negative controls in their respective groups.

25 **FIGS. 5A - 5E.** *In vitro* proliferative response of LNCs from H-2<sup>b</sup> mice immunized subcutaneously with an equimolar mixture of peptide GF56 FIG. 5A), GF57 (FIG. 5B), GF61 (FIG. 5C), GF66 (FIG. 5D) and GF51 (FIG. 5E). Recall peptide GF51 was used as a negative control. Mean proliferation with PPD positive control was  $122.0541 \times 10^3$  cpm. "NIL" represents background  
30 (no added antigen).



**FIGS. 6A - 6F.** T-proliferative response of LNCs from C57BL/6 and B10 mice immunized with an equimolar mixture of peptides GF56, GF57, and GF61 in CFA, and challenged *in vitro* with individual peptides. Each recall peptide was used at two different concentrations: 20 µg/mL (hatched bars), and 2 µg/mL (dotted bars). "NIL" represents background (no added antigen). Recall peptide GF51 was used as a negative control.

**FIG. 7.** Mapping of the minimal T-cell proliferative epitope in the GF57 peptide of HPV16E6 protein. Underlined amino acids indicate the minimal T-cell proliferative epitope.

**FIGS. 8A and 8B.** *In vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF57, and challenged with GF57 overlapping truncated peptides. Recall peptides GF57 and GF51 were used as positive and negative controls respectively. "NIL" represents background (no added antigen).

**FIG. 9.** Mapping of the minimal T-cell proliferative epitope in the GF61 peptide of HPV16E6 protein. Underlined amino acids indicate the minimal T-cell proliferative epitope.

**FIGS. 10A and 10B.** *In vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF61, and challenged with GF57 overlapping truncated peptides. Recall peptides GF61 and GF51 were used as positive and negative controls respectively. "NIL" represents background (no added antigen).

**FIGS. 11A and 11B.** *In vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF51, and challenged with GF57 overlapping truncated peptides. "NIL" represents background (no added antigen).

**FIGS. 12A and 12B.** *In vitro* proliferative response of LNCs from C57BL/6 mice immunized subcutaneously with peptide GF51, and challenged with GF61 overlapping truncated peptides. "NIL" represents background (no added antigen).

5

**FIGS. 13A - 13D.** Effect of 'VYRDGNPYA' and 'QYNKPLCDLL' T<sub>H</sub>-epitopes on the antibody response to 'EYMLD' and 'QAEPD' B-epitopes. Peptide GF101 (MHGDTPTLHEYMLDLQPE) and 8Q (QAEPDRAHYNIVTFCKCD) are HPV16 E7 peptides which contain the E7 B-epitopes EYMLD and QAEPD respectively. Used as positive controls, 8F and 6D are MAbs to EYMLD and QAEPD respectively. Sera were collected from H-2<sup>b</sup> mice before and after immunization with either VYRDGNPYA (VYR) alone, QYNKPLCDLL (QYN) alone, VYRDGNPYA linked to EYMLD and QAEPD (E-VYR-Q), QYNKPLCDLL linked to EYMLD and QAEPD (E-QYN-Q) or with a peptide consisting of EYMLD and QAEPD epitopes only (E-Q).

10

15

**FIG. 14.** Construction of recombinant baculovirus transfer vector pVLBPV1L1/Thprom-QAEPD. A hybrid nucleotide sequence encoding a modified BPV1 L1 gene in which a chimeric peptide comprising VYRDGNPYA and the B-cell epitope QAEPD, is fused to the C-terminus of the BPV1 L1 sequence by PCR amplification. This fusion results in deletion of nucleotides from 7625 downstream of the BPV1 wild-type nucleotide sequence.

20

CLAIMS

What is claimed is:

1. A peptide comprising the sequence of amino acids VYRDGNPYA (SEQ ID NO 1) inclusive of a single amino acid deletion, substitution or addition made therein.
2. A peptide homolog of the peptide according to claim 1.
3. A peptide of 10 to 34 amino acids corresponding to a natural sequence of amino acids encoded by HPV16 E6 or homologous sequence thereof which natural or homologous sequence comprises the sequence of amino acids defined by the peptide of claim 1 or claim 2.
4. A peptide of 13 to 20 amino acids corresponding to a natural sequence of amino acids encoded by HPV16 E6 or homologous sequence thereof which natural or homologous sequence comprises the sequence of amino acids defined by the peptide of claim 1 or claim 2.
5. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope.
6. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein said construct comprises between 10 and 34 amino acids.
7. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences

encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope wherein, said construct comprises between 13 and 20 amino acids.

8. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is from a pathogenic organism.

9. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is from a papillomavirus.

10. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is from HPV16 E7.

11. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is QAEPD or EYMLD.

12. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each CTL epitope is from a tumor or viral source.

13. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each CTL epitope is from a papillomavirus.

14. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each CTL epitope is from HPV16 E6 or HPV16 E7.

15. An immunogenic composition comprising a peptide according to claim 1 or claim 2 in combination with an adjuvant and/or a delivery vehicle.

16. An immunogenic composition comprising a peptide according to claim 1 or claim 2 in combination with an adjuvant and/or a delivery vehicle, wherein said delivery vehicle comprises a chimeric VLP including a modified viral capsid protein having said peptide fused thereto.

17. An immunogenic composition comprising a peptide according to claim 1 or claim 2 in combination with an adjuvant and/or a delivery vehicle, wherein said delivery vehicle comprises a chimeric VLP including a

modified papillomavirus capsid protein having said peptide fused thereto.

18. An immunogenic composition comprising a chimeric peptide construct in combination with an adjuvant and/or a delivery vehicle, said chimeric peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope.

19. An immunogenic composition comprising a chimeric peptide construct in combination with an adjuvant and/or a delivery vehicle, said chimeric peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein said delivery vehicle comprises a chimeric VLP including a modified viral capsid protein having said chimeric peptide construct fused thereto.

20. An immunogenic composition comprising a chimeric peptide construct in combination with an adjuvant and/or a delivery vehicle, said chimeric peptide construct comprising a peptide according to claim 1 or claim 2 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein said delivery vehicle comprises a chimeric VLP including a modified papillomavirus capsid protein having said chimeric peptide construct fused thereto.

21. A peptide comprising the sequence of amino acids QYNKPLCDLL (SEQ ID NO 2) inclusive of a single amino acid deletion, substitution or addition made therein.

22. A peptide homolog of the peptide according to claim 21.

23. A peptide of 10 to 34 amino acids corresponding to a natural sequence of amino acids encoded by HPV16 E6 or homologous sequence thereof which natural or homologous sequence comprises the sequence of amino acids defined by the peptide of claim 21 or claim 22.

24. A peptide of 13 to 20 amino acids corresponding to a natural sequence of amino acids encoded by HPV16 E6 or homologous sequence thereof which natural or homologous sequence comprises the sequence of amino acids defined by the peptide of claim 21 or claim 22.

5 25. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid  
10 sequences encoding the or each CTL epitope.

26. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences  
15 encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein said construct comprises between 10 and 34 amino acids.

27. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL  
20 epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope wherein, said construct comprises between 13 and 20 amino acids.

25 28. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid  
30 sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is from a pathogenic organism.

29. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is from a papillomavirus.
30. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is from HPV16 E7.
31. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each B-cell epitope is QAEPD or EYMLD.
32. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each CTL epitope is from a tumor or viral source.
33. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21



or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein the or each CTL epitope is from a papillomavirus.

- 5 34. A chimeric peptide construct for generating an immune response against one or more B-cell epitopes and/or one or more CTL epitopes said peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid  
10 sequences encoding the or each CTL epitope, wherein the or each CTL epitope is from HPV16 E6 or HPV16 E7.
35. An immunogenic composition comprising a peptide according to claim 21 or claim 22 in combination with an adjuvant and/or a delivery vehicle.
- 15 36. An immunogenic composition comprising a peptide according to claim 21 or claim 22 in combination with an adjuvant and/or a delivery vehicle, wherein said delivery vehicle comprises a chimeric VLP including a modified viral capsid protein having said peptide fused thereto.
37. An immunogenic composition comprising a peptide according to claim 21 or claim 22 in combination with an adjuvant and/or a delivery  
20 vehicle, wherein said delivery vehicle comprises a chimeric VLP including a modified papillomavirus capsid protein having said peptide fused thereto.
38. An immunogenic composition comprising a chimeric peptide construct in combination with an adjuvant and/or a delivery vehicle, said  
25 chimeric peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope.
39. An immunogenic composition comprising a chimeric peptide  
30 construct in combination with an adjuvant and/or a delivery vehicle, said chimeric peptide construct comprising a peptide according to claim 21 or

claim 22 linked directly or indirectly to one or more amino acid sequences encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein said delivery vehicle comprises a chimeric VLP including a modified viral capsid protein having said chimeric peptide construct fused thereto.

- 5 40. An immunogenic composition comprising a chimeric peptide construct in combination with an adjuvant and/or a delivery vehicle, said chimeric peptide construct comprising a peptide according to claim 21 or claim 22 linked directly or indirectly to one or more amino acid sequences
- 10 encoding the or each B-cell epitope and/or one or more amino acid sequences encoding the or each CTL epitope, wherein said delivery vehicle comprises a chimeric VLP including a modified papillomavirus capsid protein having said chimeric peptide construct fused thereto.

1/25

PEPTIDE	SEQUENCE	LENGTH
GF 51	<sup>1</sup> MHQKRTAMFQDPQERPRKLPQLC <sup>23</sup>	(23aa)
GF 52	<sup>14</sup> RPRKLPQLCTELQTTIHD <sup>32</sup>	(18aa)
GF53	<sup>24</sup> TELQTTIHDIIIECVYCK <sup>41</sup>	(18aa)
GF54	<sup>32</sup> DIIECVYCKQQLLRREVYD <sup>51</sup>	(20aa)
GF55	<sup>42</sup> QQLLRREVYDFAFRDL <sup>57</sup>	(16aa)
GF56	<sup>51</sup> DFAFRDLCIVYRDGNPYA <sup>68</sup>	(18aa)
GF57	<sup>58</sup> CIVYRDGNPYAVCDKCLKFY <sup>77</sup>	(20aa)
GF58	<sup>69</sup> VCDKCLKFYISKISEYRHY <sup>86</sup>	(18aa)
GF59	<sup>78</sup> SKISEYRHYCYSLYGTTL <sup>95</sup>	(18aa)
GF60	<sup>87</sup> CYSLYGTTLQQYNKPLC <sup>104</sup>	(18aa)
GF61	<sup>96</sup> EQQYNKPLCDLLIRCINC <sup>113</sup>	(18aa)
GF62	<sup>105</sup> DLLIRCINCQKPLCPEEK <sup>122</sup>	(18aa)
GF63	<sup>114</sup> QKPLCPEEKQRHLDKKQRF <sup>132</sup>	(19aa)
GF64	<sup>123</sup> QRHLDKKQRFHNIRGRWT <sup>140</sup>	(18aa)
GF65	<sup>132</sup> FHNIRGRWTGRCMSCCRS <sup>149</sup>	(18aa)
GF66	<sup>141</sup> GRCMSCCRSSRTRPETQL <sup>158</sup>	(18aa)

FIG. 1

2/25

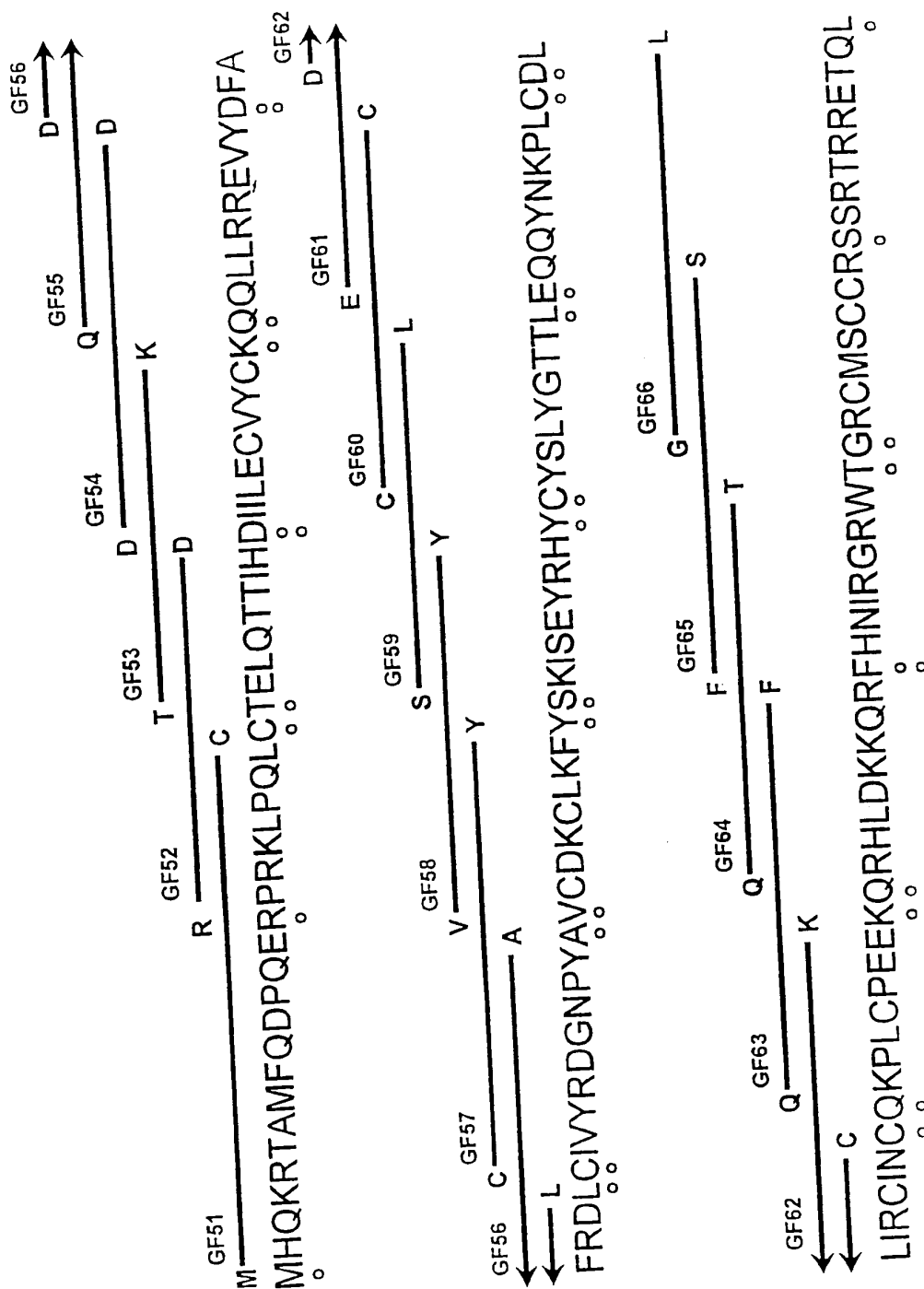


FIG. 2

3/25

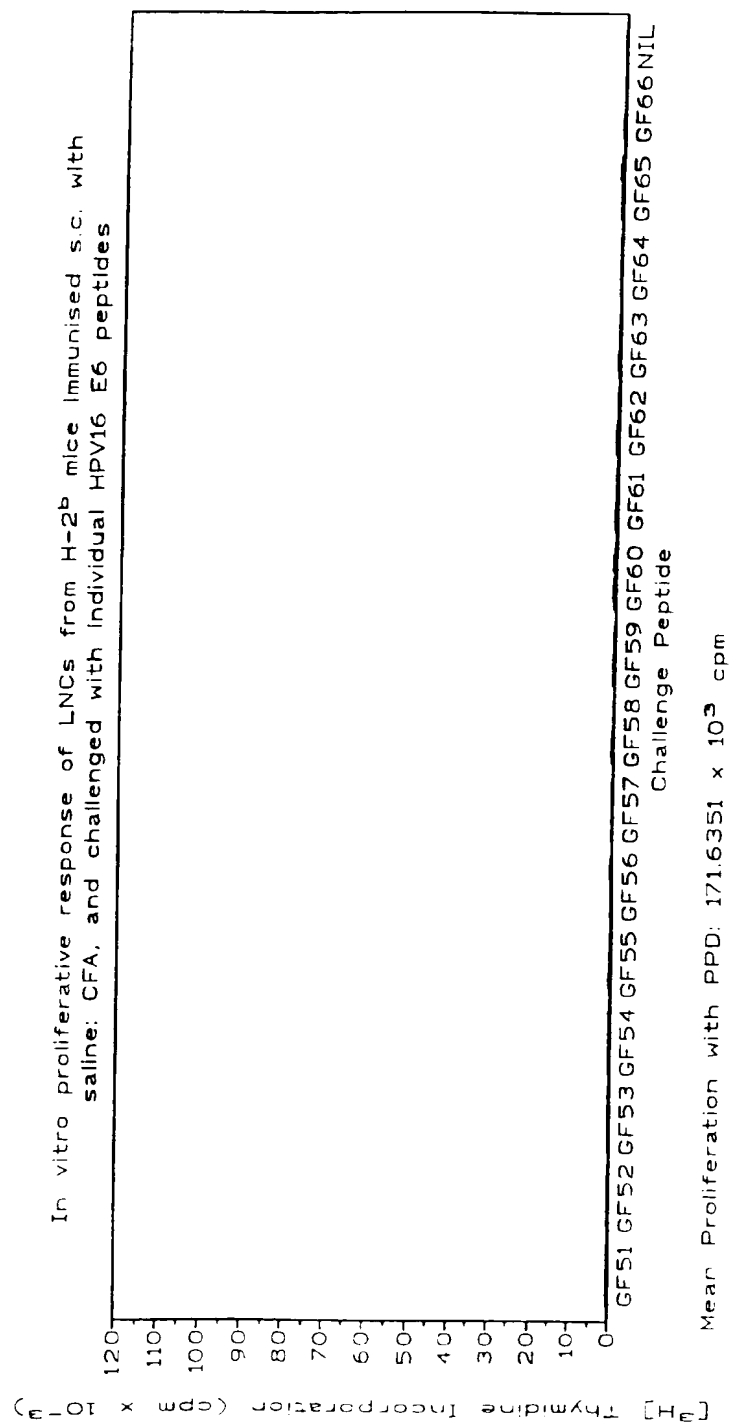


FIG. 3A

4/25

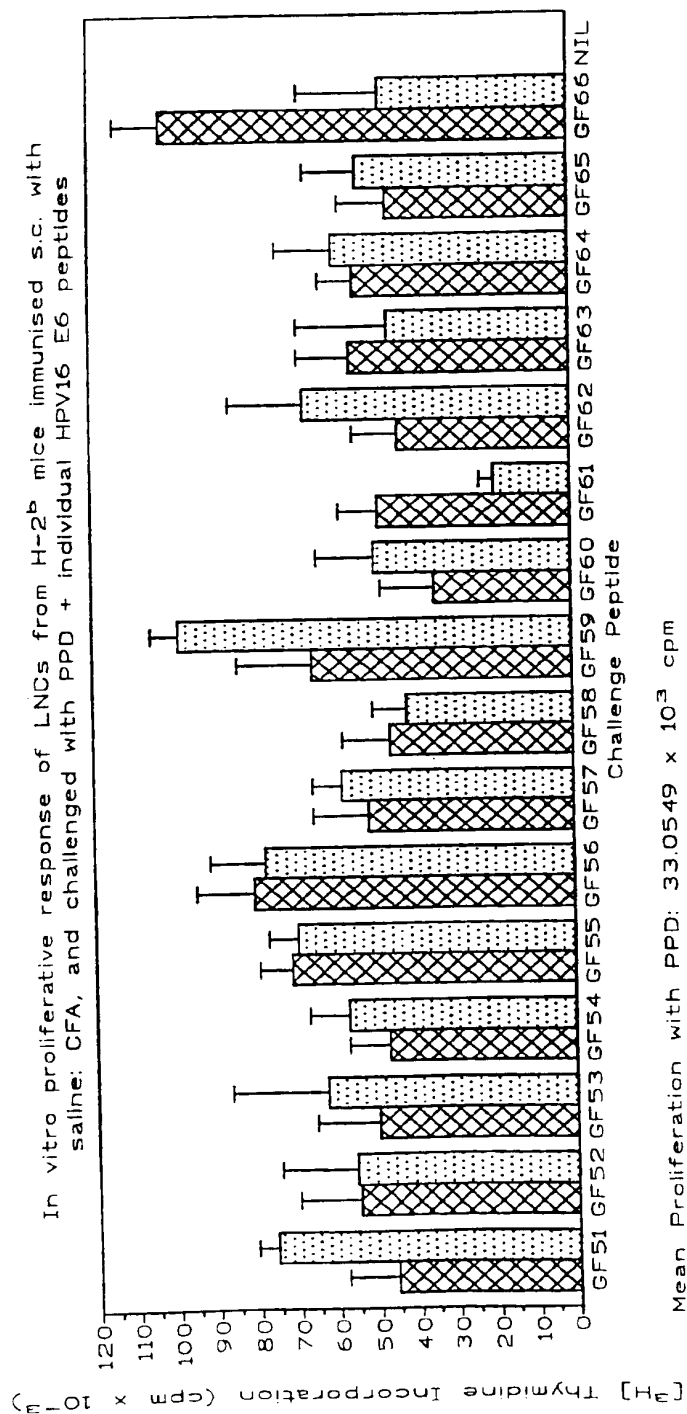


FIG. 3B

5/25

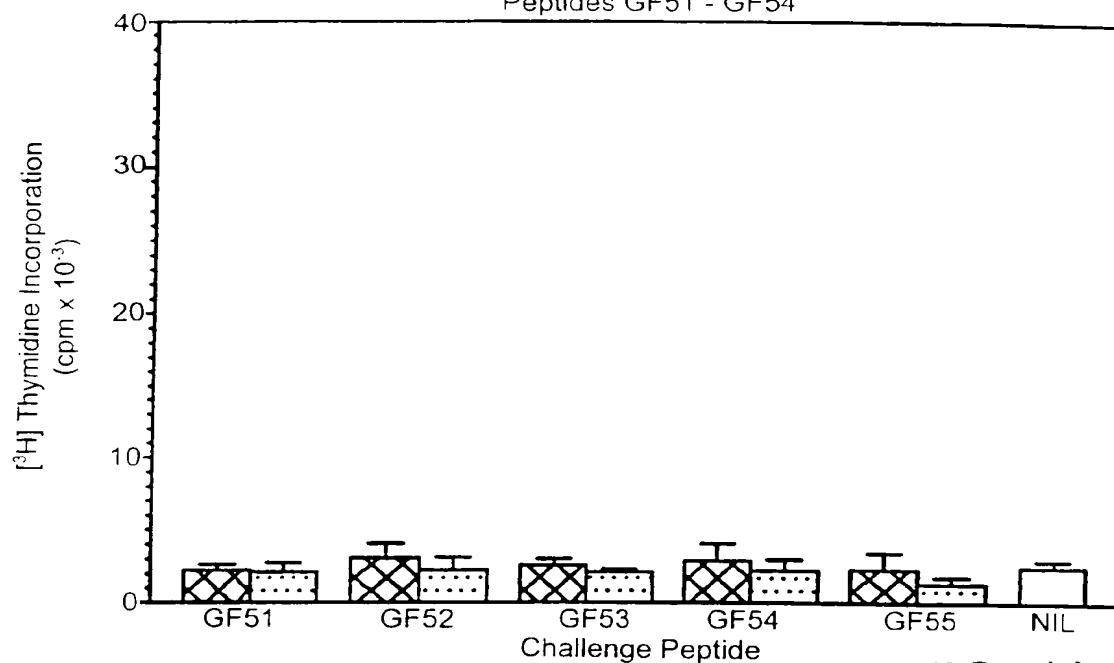
In Vitro Proliferative Response of LNCs from Mice immunised with  
Peptides GF51 - GF54Mean Proliferation with PPD:  $74.7195 \times 10^3$  cpm

FIG. 4A

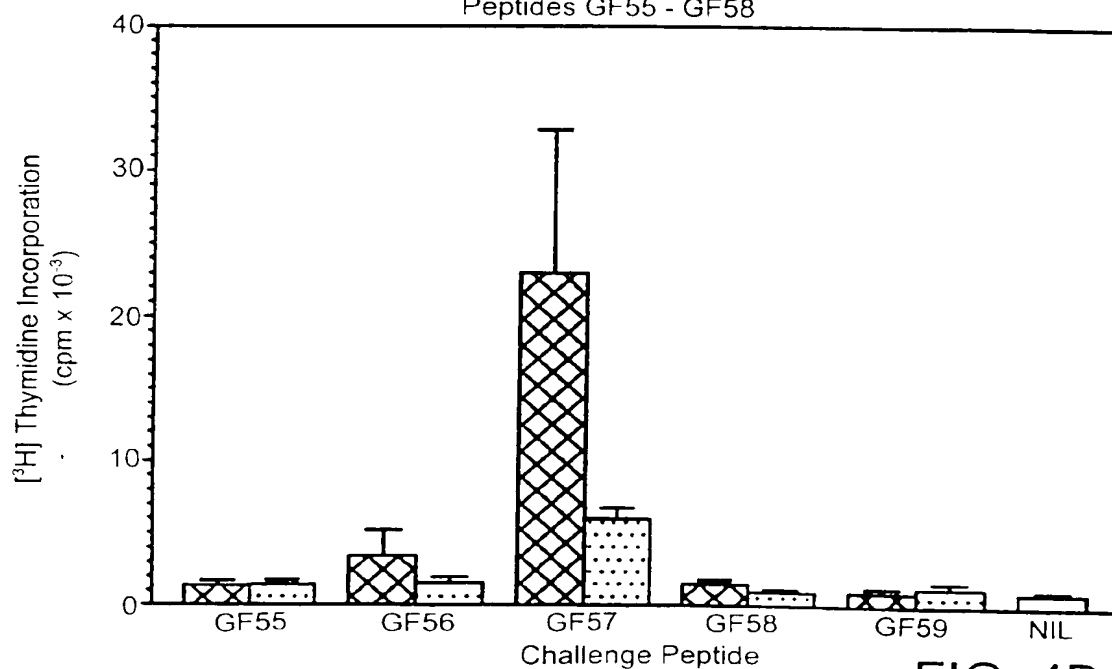
In Vitro Proliferative Response of LNCs from Mice immunised with  
Peptides GF55 - GF58Mean Proliferation with PPD:  $80.2908 \times 10^3$  cpm

FIG. 4B

6/25

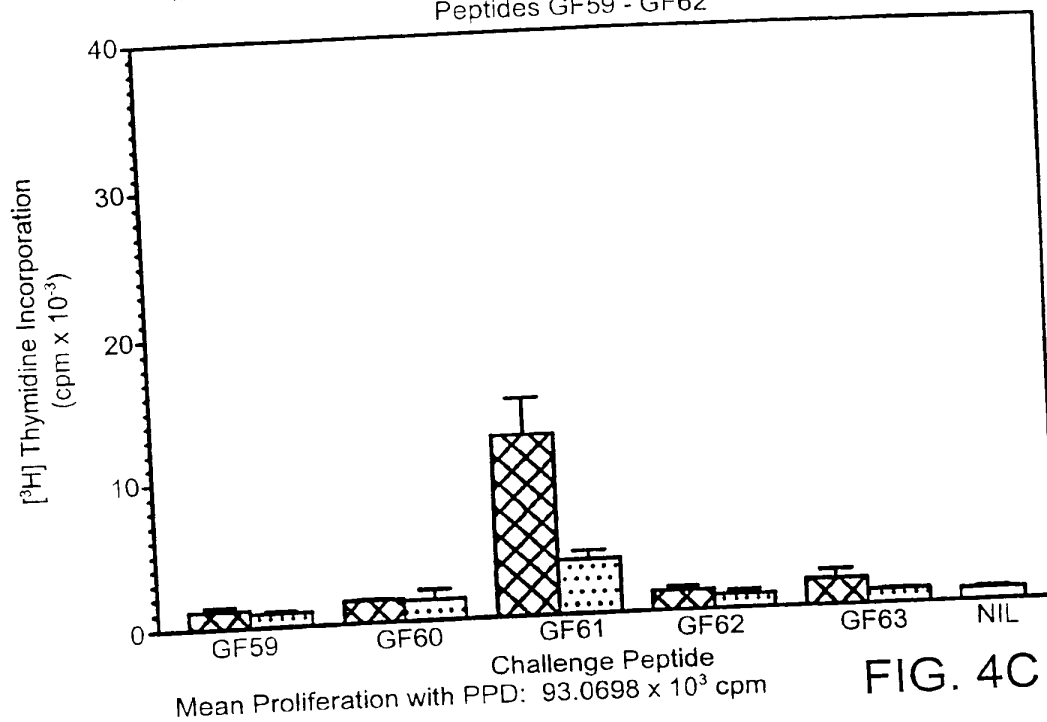
In Vitro Proliferative Response of LNCs from Mice immunised with  
Peptides GF59 - GF62

FIG. 4C

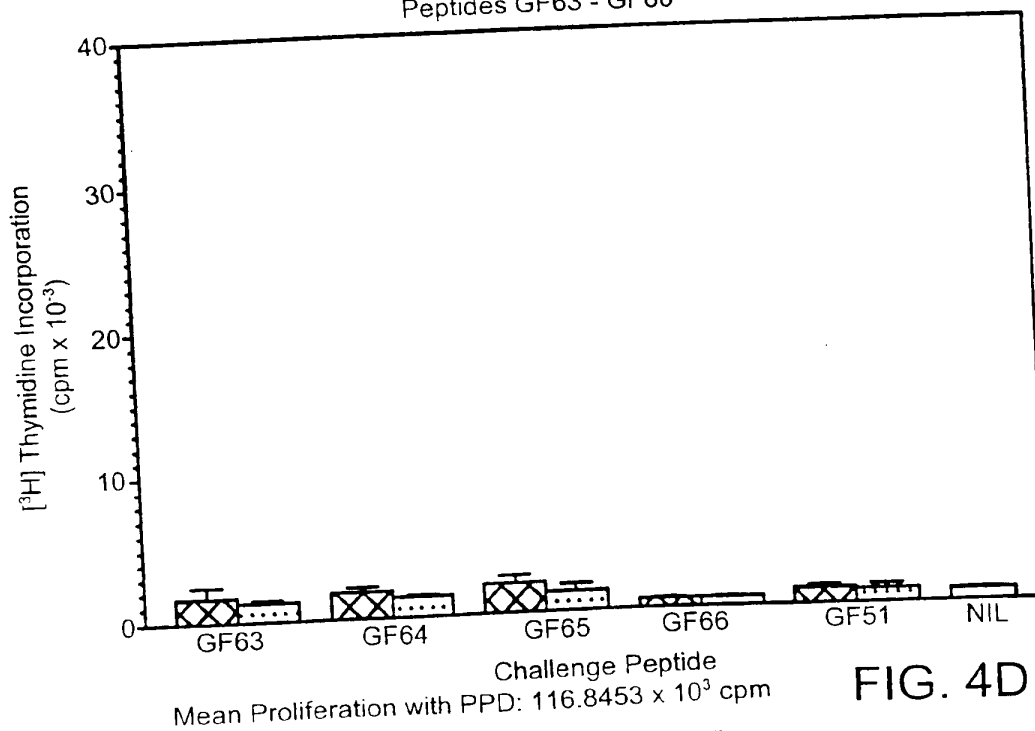
In Vitro Proliferative Response of LNCs from Mice immunised with  
Peptides GF63 - GF66

FIG. 4D



7/25

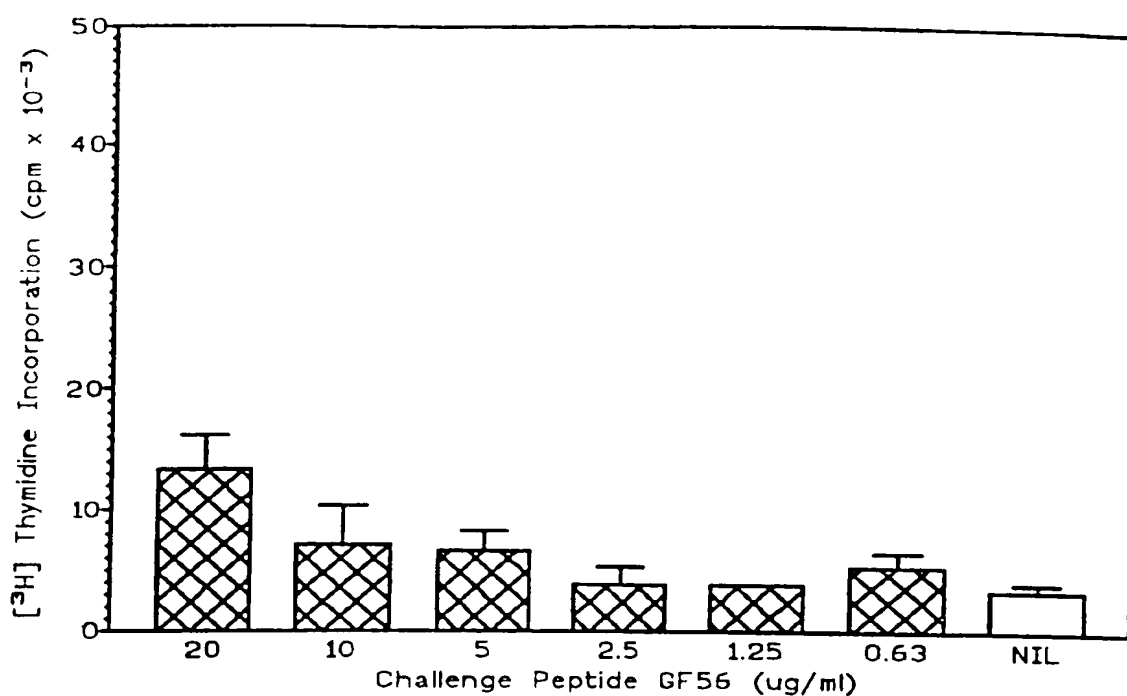


FIG. 5A

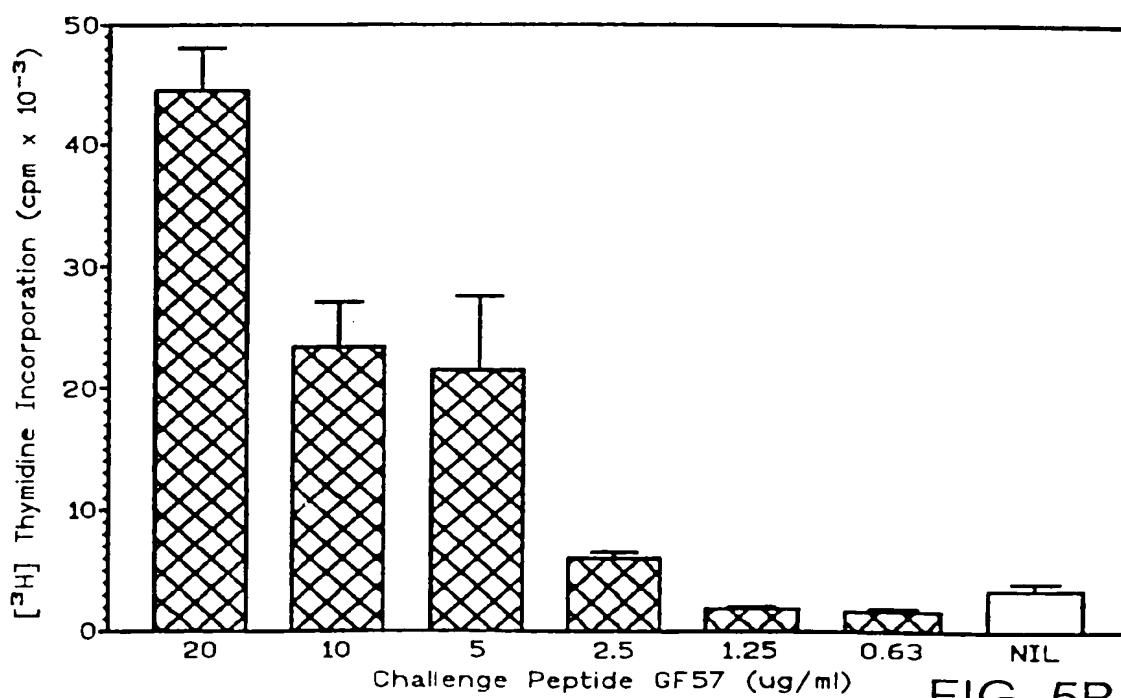


FIG. 5B

8/25

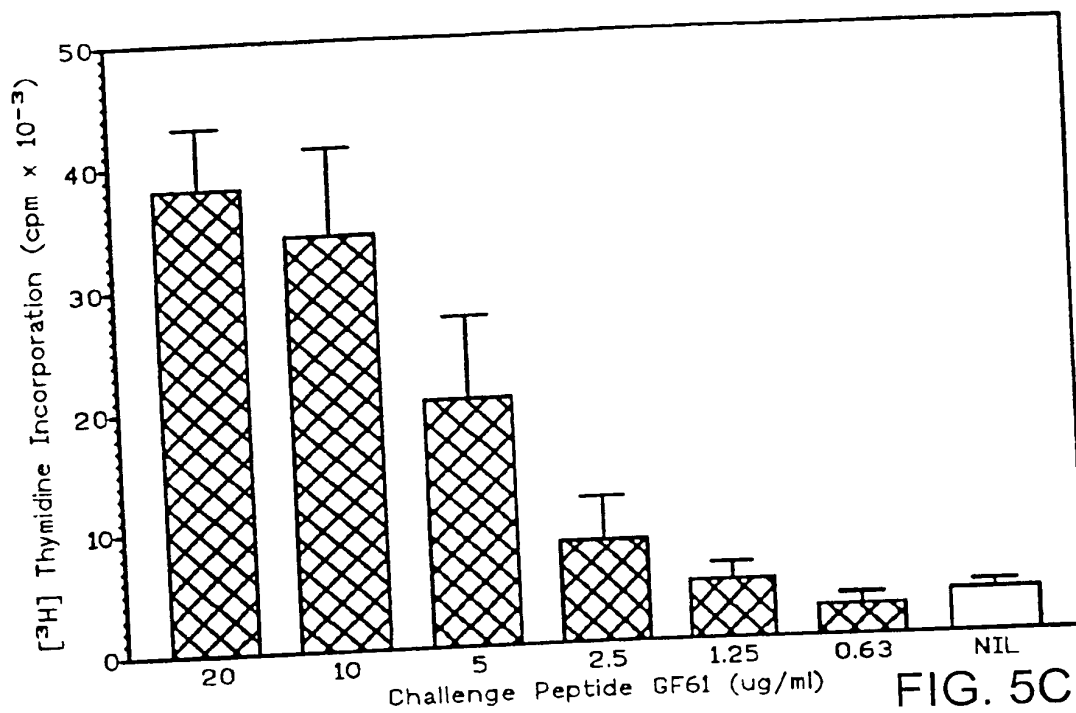


FIG. 5C

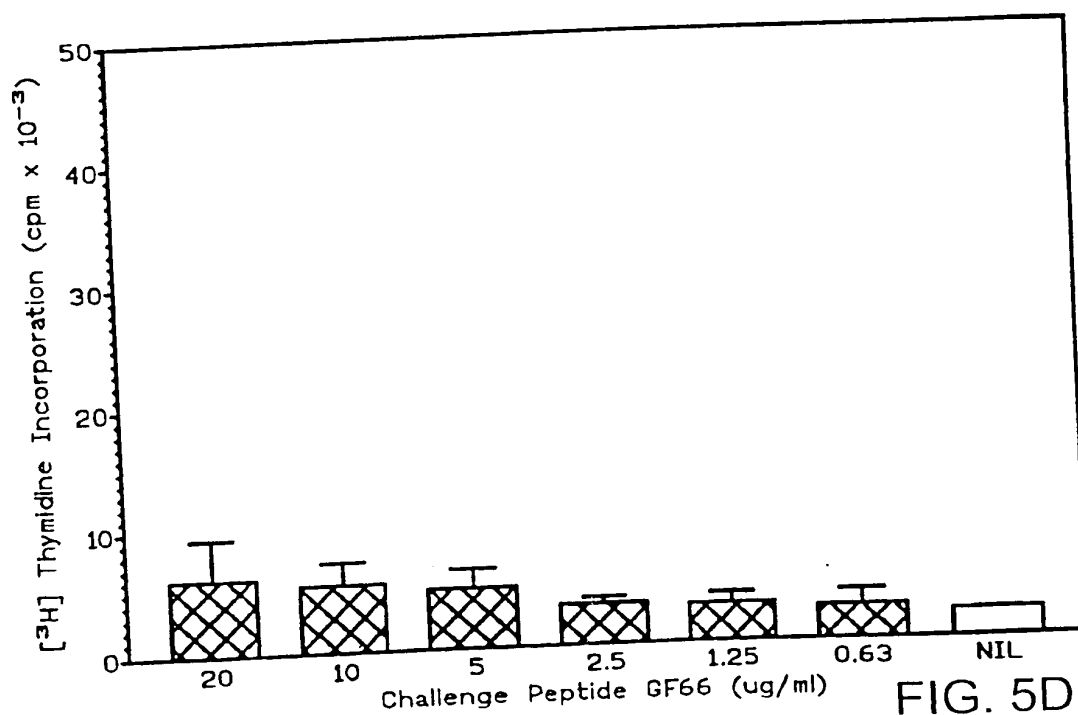


FIG. 5D

9/25

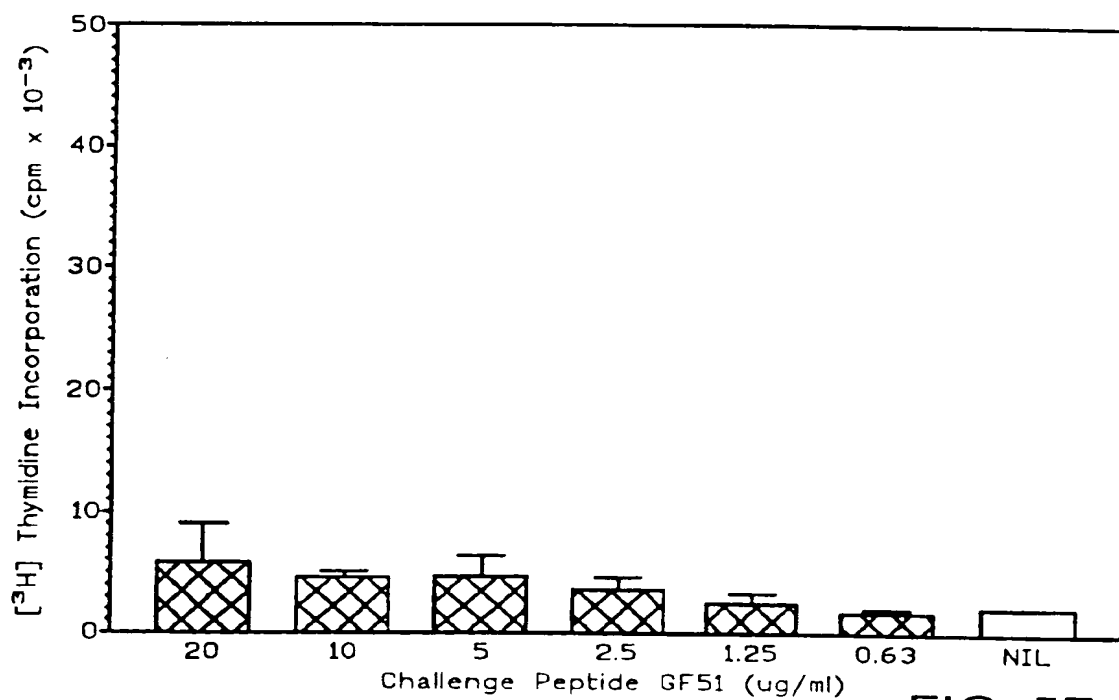


FIG. 5E

10/25

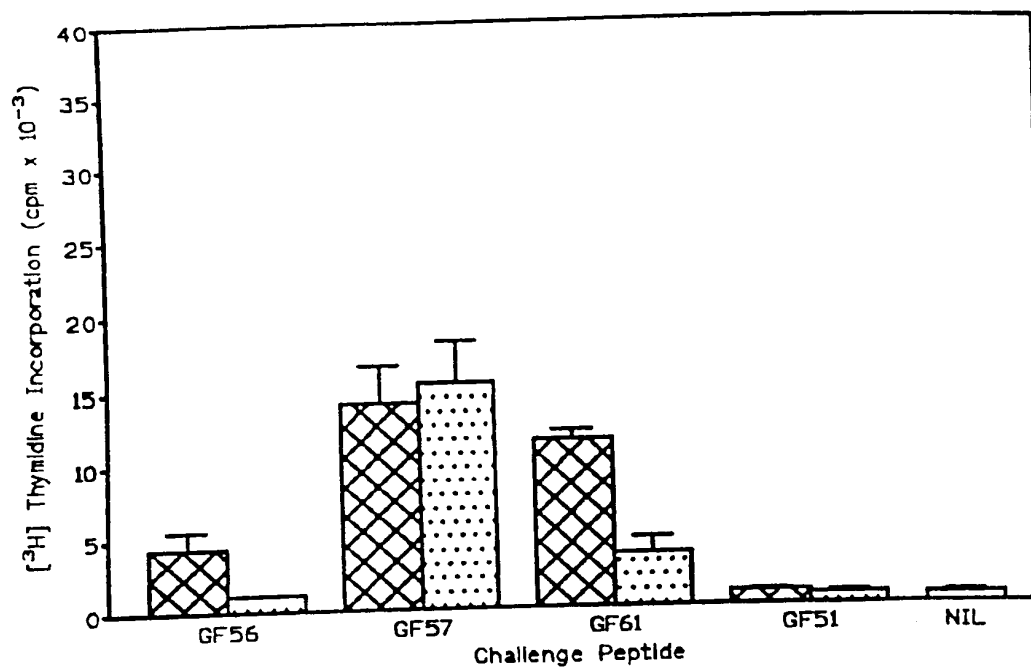
Mean Proliferation with PPD:  $98.4109 \times 10^3$  cpm

FIG. 6A

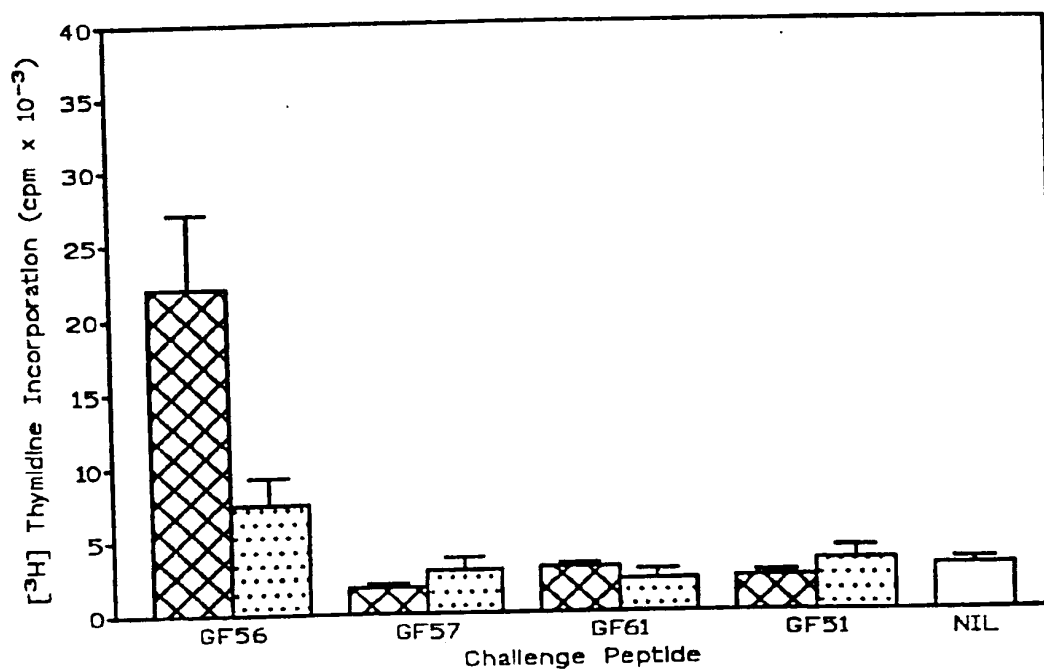
Mean Proliferation with PPD:  $146.7829 \times 10^3$  cpm

FIG. 6B

11/25

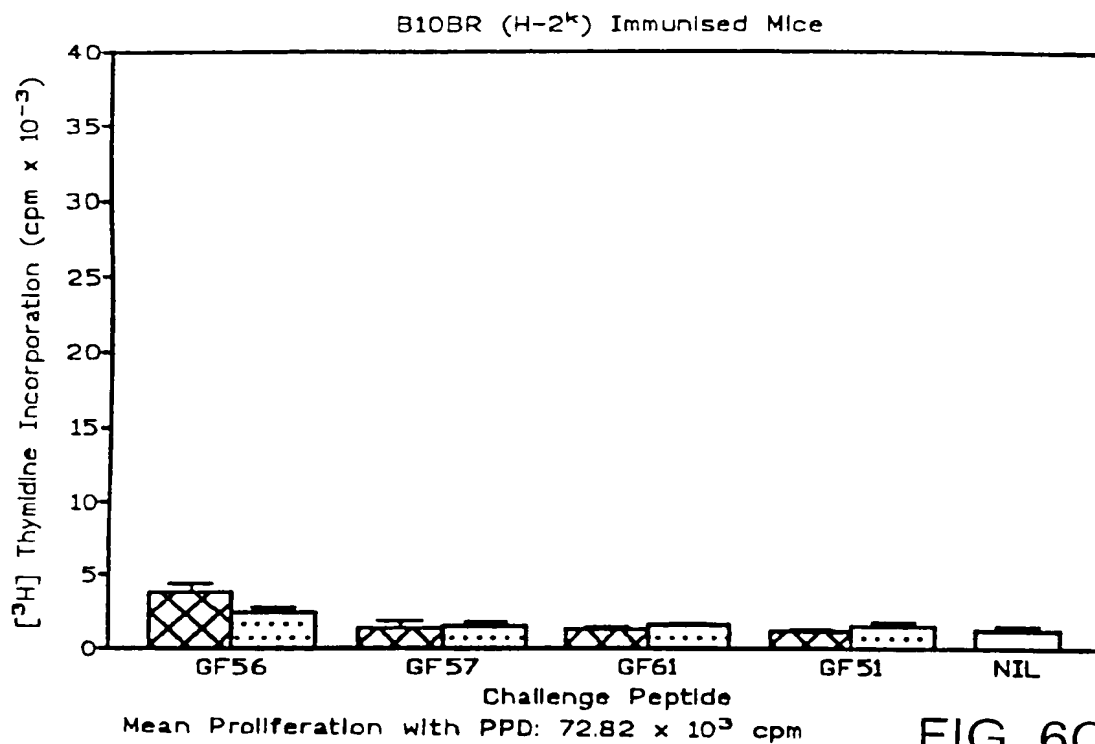


FIG. 6C

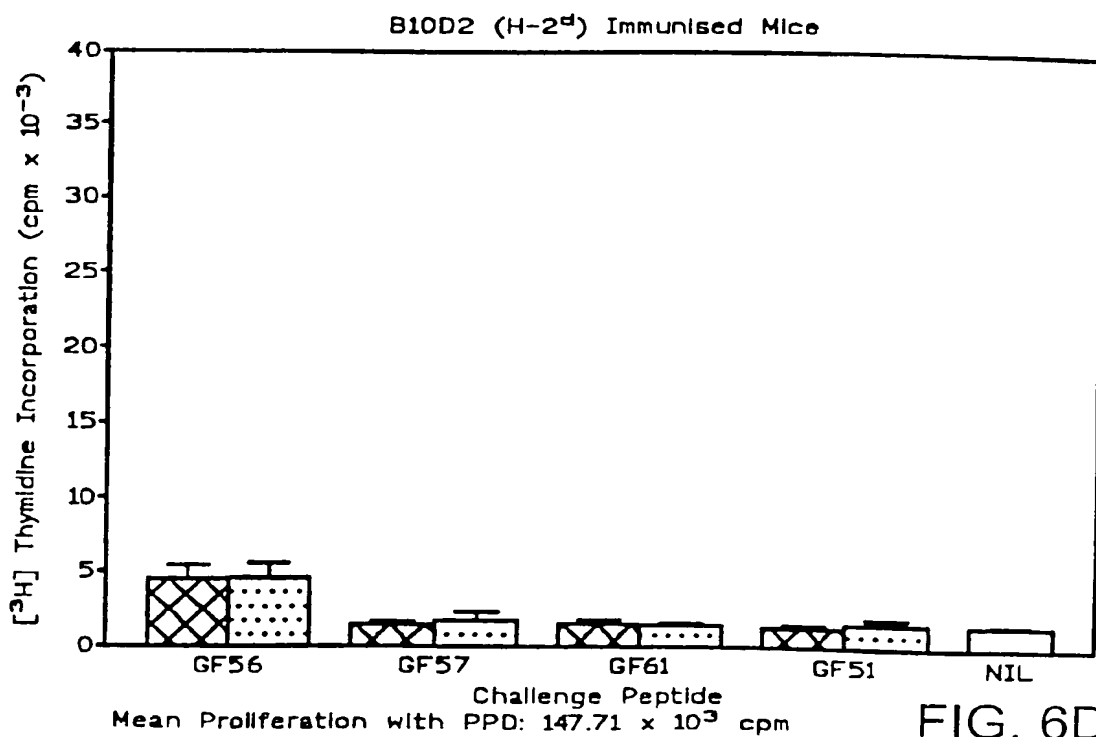


FIG. 6D

12/25

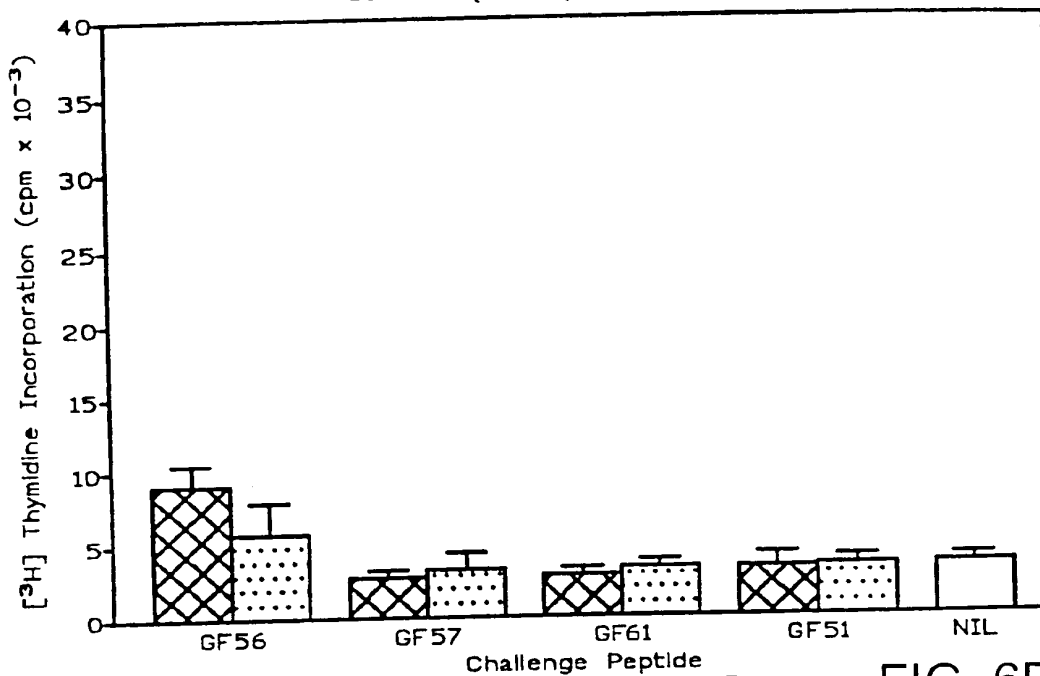
B10A.2R (H-2<sup>b2</sup>) Immunised MiceMean Proliferation with PPD: 165.0767 x 10<sup>3</sup> cpm

FIG. 6E

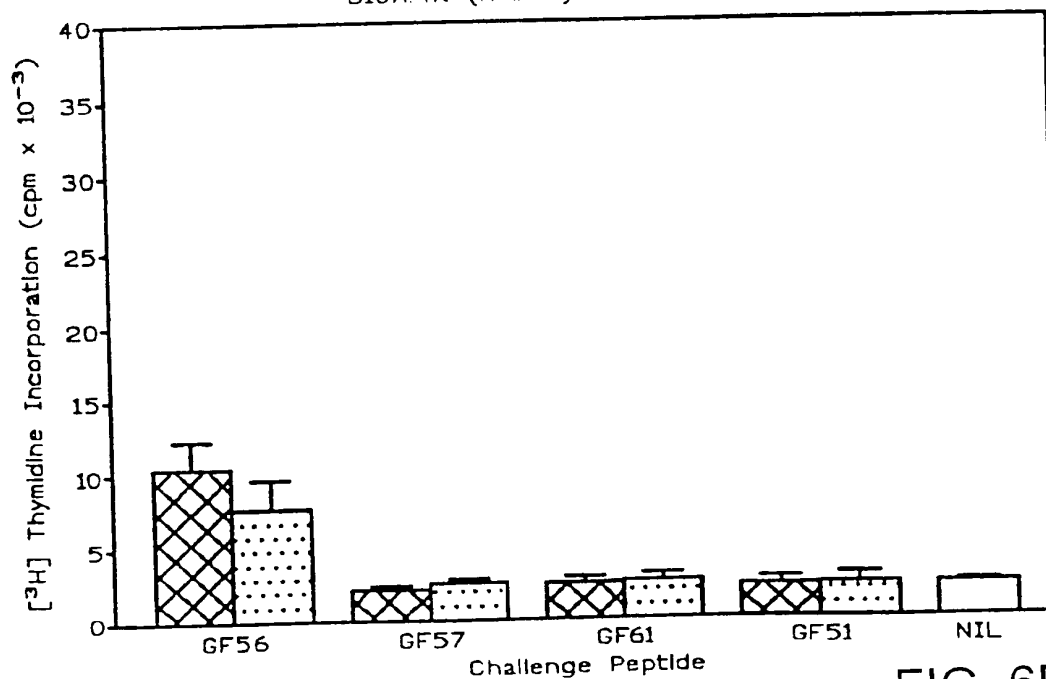
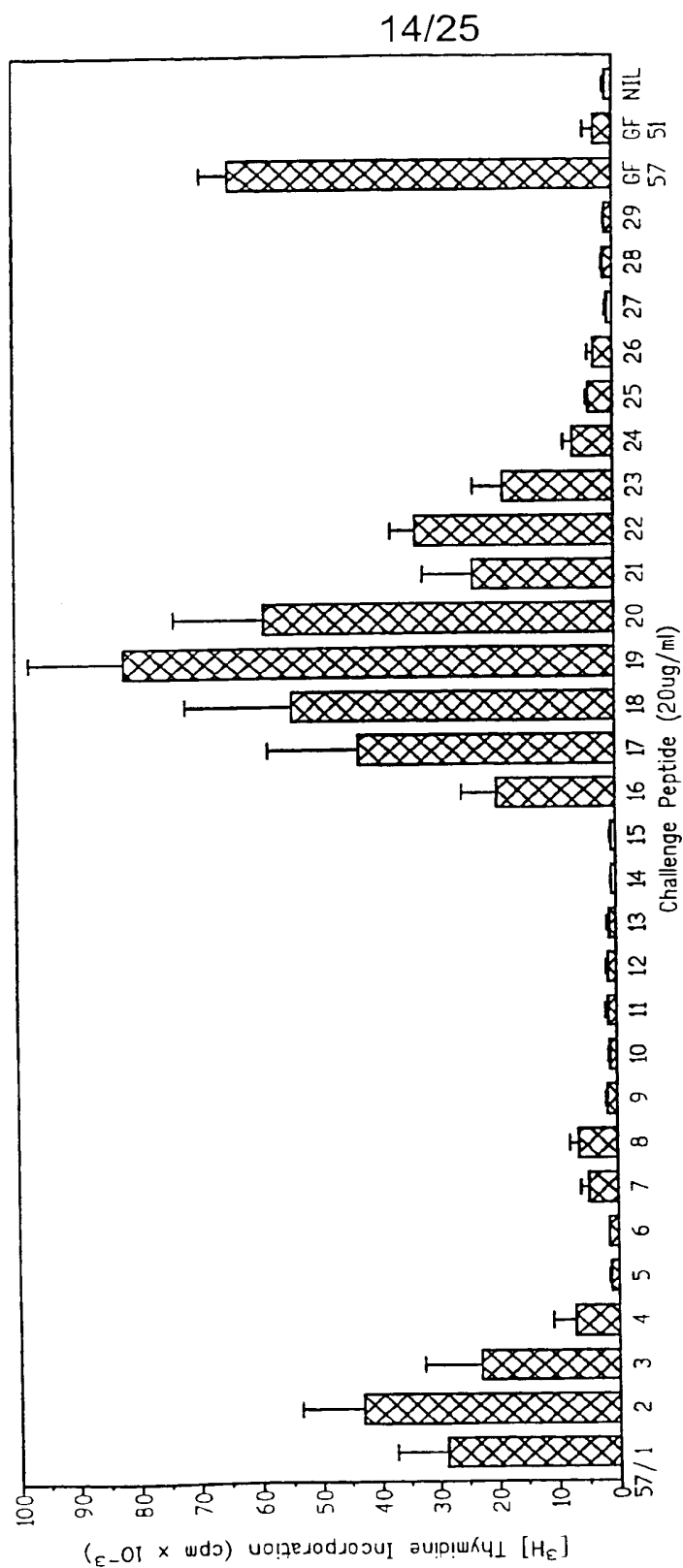
B10A.4R (H-2<sup>b4</sup>) Immunised MiceMean Proliferation with PPD: 207.967 x 10<sup>3</sup> cpm

FIG. 6F

13/25

PEPTIDE 57/1.	CIVYRDGNPYAVCDKCLKFY	(20 aa)
PEPTIDE 57/2.	IVYRDGNPYAVCDKCLKFY	(19 aa)
PEPTIDE 57/3.	VYRDGNPYAVCDKCLKFY	(18 aa)
PEPTIDE 57/4.	YRDGNPYAVCDKCLKFY	(17 aa)
PEPTIDE 57/5.	RDGNPYAVCDKCLKFY	(16 aa)
PEPTIDE 57/6.	DGNPYAVCDKCLKFY	(15 aa)
PEPTIDE 57/7.	GNPYAVCDKCLKFY	(14 aa)
PEPTIDE 57/8.	NPYAVCDKCLKFY	(13 aa)
PEPTIDE 57/9.	PYAVCDKCLKFY	(12 aa)
PEPTIDE 57/10.	YAVCDKCLKFY	(11 aa)
PEPTIDE 57/11.	AVCDKCLKFY	(10 aa)
PEPTIDE 57/12.	VCDKCLKFY	(09 aa)
PEPTIDE 57/13.	CDKCLKFY	(08 aa)
PEPTIDE 57/14.	DKCLKFY	(07 aa)
PEPTIDE 57/15.	KCLKFY	(06 aa)
PEPTIDE 57/16.	CIVYRDGNPYAVCDKCLKF	(19 aa)
PEPTIDE 57/17.	CIVYRDGNPYAVCDKCLK	(18 aa)
PEPTIDE 57/18.	CIVYRDGNPYAVCDKCL	(17 aa)
PEPTIDE 57/19.	CIVYRDGNPYAVCDKC	(16 aa)
PEPTIDE 57/20.	CIVYRDGNPYAVCDK	(15 aa)
PEPTIDE 57/21.	CIVYRDGNPYAVCD	(14 aa)
PEPTIDE 57/22.	CIVYRDGNPYAVC	(13 aa)
PEPTIDE 57/23.	CIVYRDGNPYAV	(12 aa)
PEPTIDE 57/24.	CIVYRDGNPYA	(11 aa)
PEPTIDE 57/25.	CIVYRDGNPY	(10 aa)
PEPTIDE 57/26.	CIVYRDGNP	(09 aa)
PEPTIDE 57/27.	CIVYRDGN	(08 aa)
PEPTIDE 57/28.	CIVYRDG	(07 aa)
PEPTIDE 57/29.	CIVYRD	(06 aa)

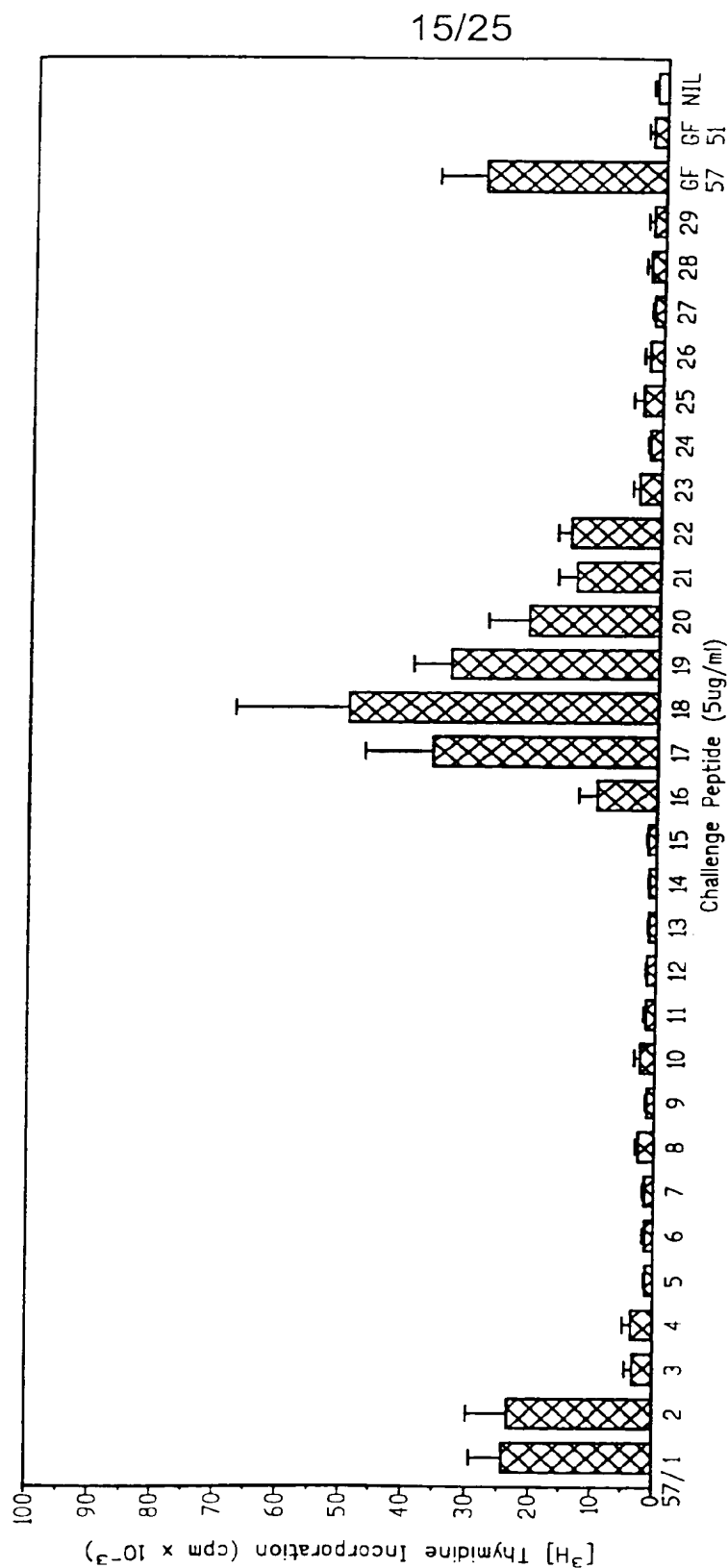
FIG. 7



Mean Proliferation with PPD: 128.1808 x 10<sup>3</sup> cpm

FIG. 8A





Mean Proliferation with PPD: 127.33665 x 10<sup>3</sup> cpm

FIG. 8B

16/25

PEPTIDE 61/1.	EQQYNKPLCDLLIRCINC	(18 aa)
PEPTIDE 61/2.	QQYNKPLCDLLIRCINC	(17 aa)
PEPTIDE 61/3.	QYNKPLCDLLIRCINC	(16 aa)
PEPTIDE 61/4.	YNKPLCDLLIRCINC	(15 aa)
PEPTIDE 61/5.	NKPLCDLLIRCINC	(14 aa)
PEPTIDE 61/6.	KPLCDLLIRCINC	(13 aa)
PEPTIDE 61/7.	PLCDLLIRCINC	(12 aa)
PEPTIDE 61/8.	LCDLLIRCINC	(11 aa)
PEPTIDE 61/9.	CDLLIRCINC	(10 aa)
PEPTIDE 61/10.	DLLIRCINC	(09 aa)
PEPTIDE 61/11.	LLIRCINC	(08 aa)
PEPTIDE 61/12.	LIRCINC	(07 aa)
PEPTIDE 61/13.	IRCINC	(06 aa)
PEPTIDE 61/14.	EQQYNKPLCDLLIRCIN	(17 aa)
PEPTIDE 61/15.	EQQYNKPLCDLLIRCI	(16 aa)
PEPTIDE 61/16.	EQQYNKPLCDLLIRC	(15 aa)
PEPTIDE 61/17.	EQQYNKPLCDLLIR	(14 aa)
PEPTIDE 61/18.	EQQYNKPLCDLLI	(13 aa)
PEPTIDE 61/19.	EQQYNKPLCDLL	(12 aa)
PEPTIDE 61/20.	EQQYNKPLCDL	(11 aa)
PEPTIDE 61/21.	EQQYNKPLCD	(10 aa)
PEPTIDE 61/22.	EQQYNKPLC	(09 aa)
PEPTIDE 61/23.	EQQYNKPL	(08 aa)
PEPTIDE 61/24.	EQQYNKP	(07 aa)
PEPTIDE 61/25.	EQQYNK	(06 aa)

FIG. 9

17/25

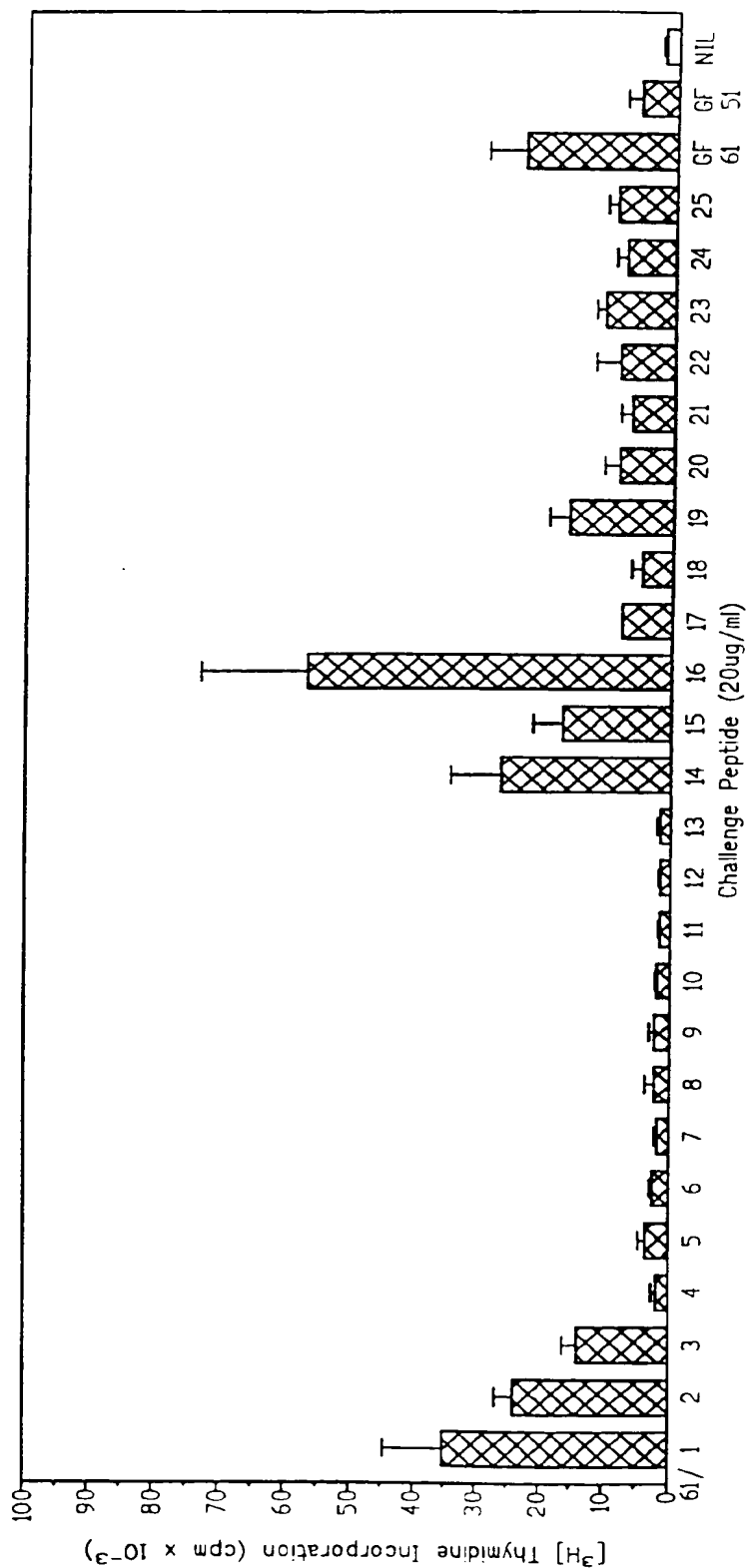
Mean Proliferation with PPD: 123.6524 x 10<sup>3</sup> cpm

FIG. 10A

18/25

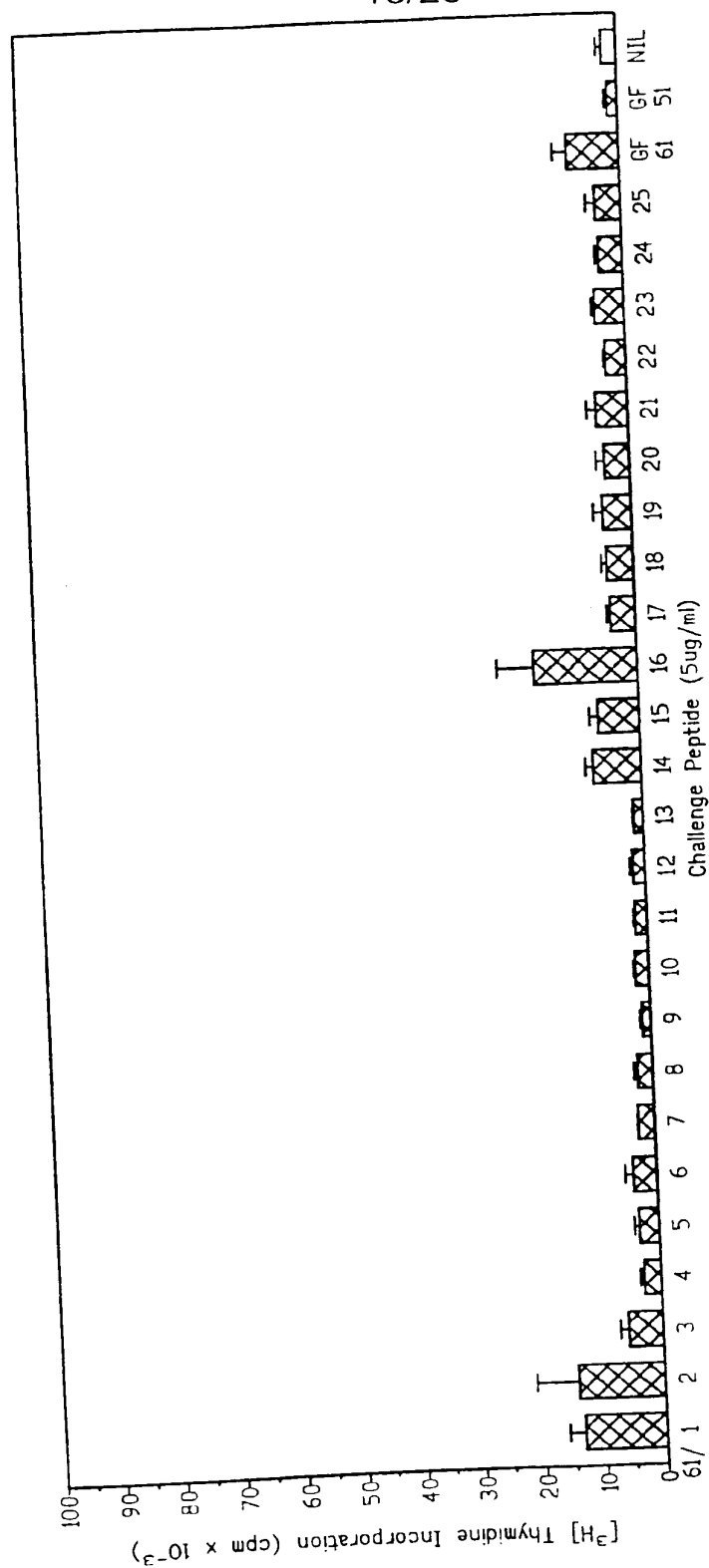
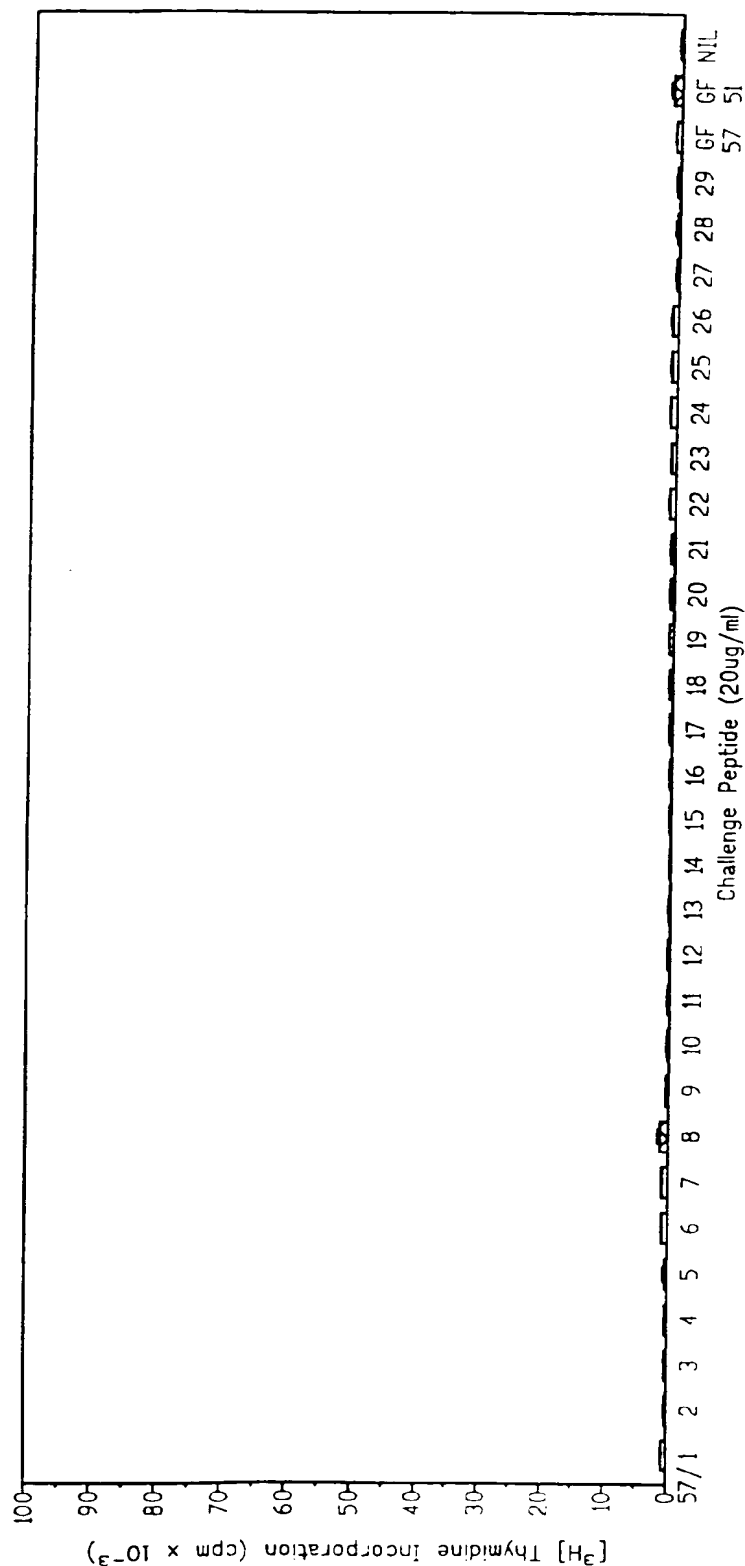
Mean Proliferation with PPD: 142.2483 x 10<sup>3</sup> cpm

FIG. 10B

19/25



Mean Proliferation with PPD: 86.9633 x 10<sup>3</sup> cpm

FIG. 11A

20/25

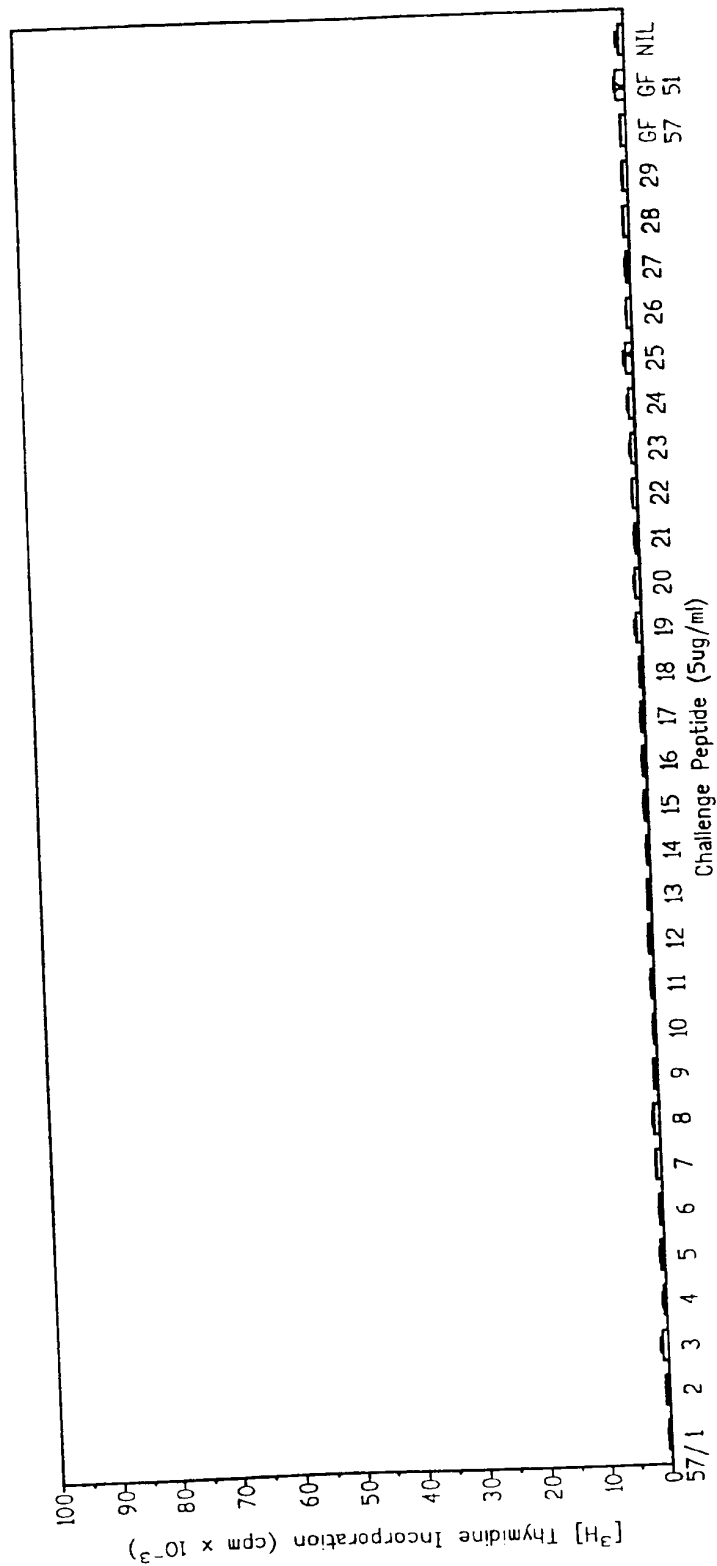
Mean Proliferation with PPD: 95.7213 x 10<sup>3</sup> cpm

FIG. 11B

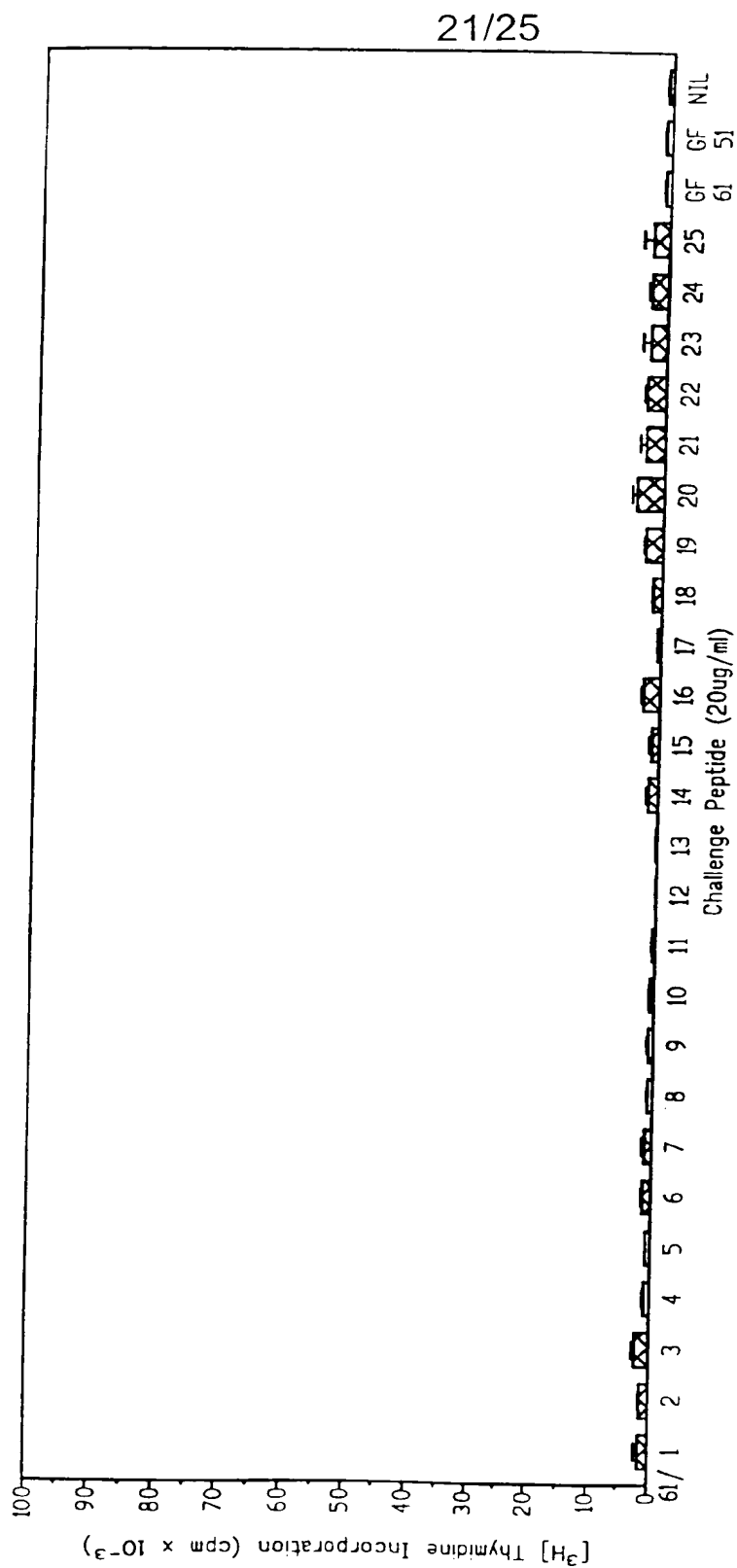


FIG. 12A

22/25

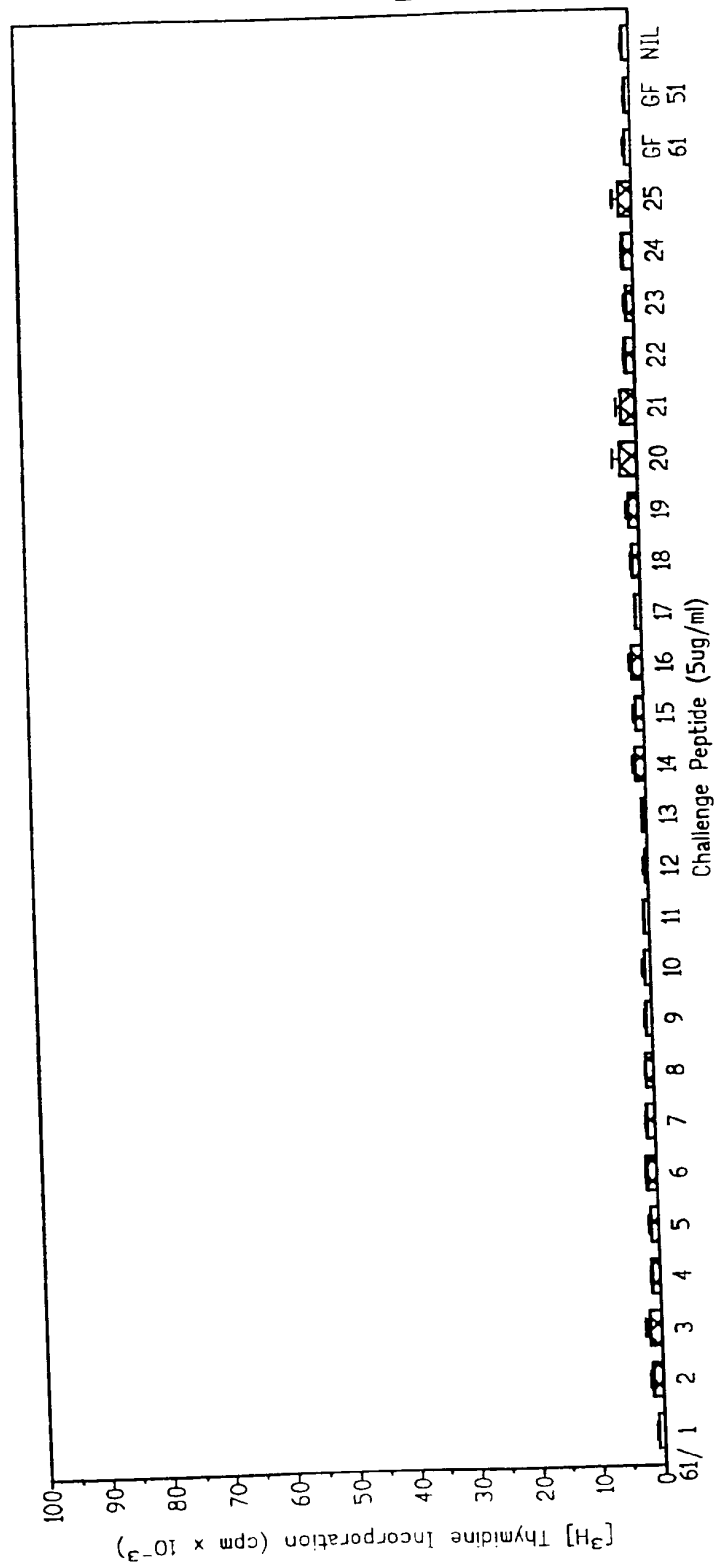


FIG. 12B



23/25

Peptide GF101 ELISA

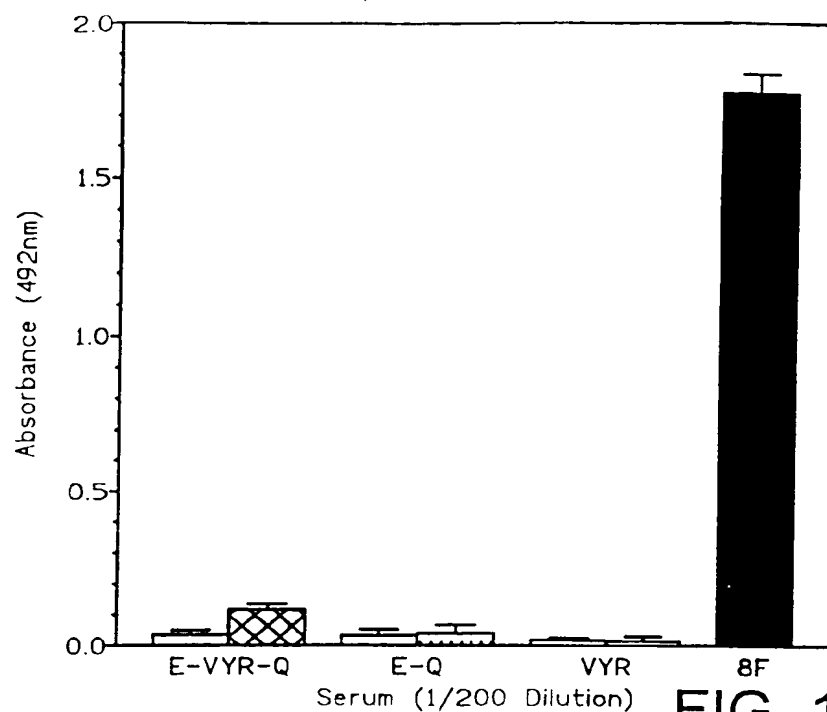


FIG. 13A

Peptide GF101 ELISA

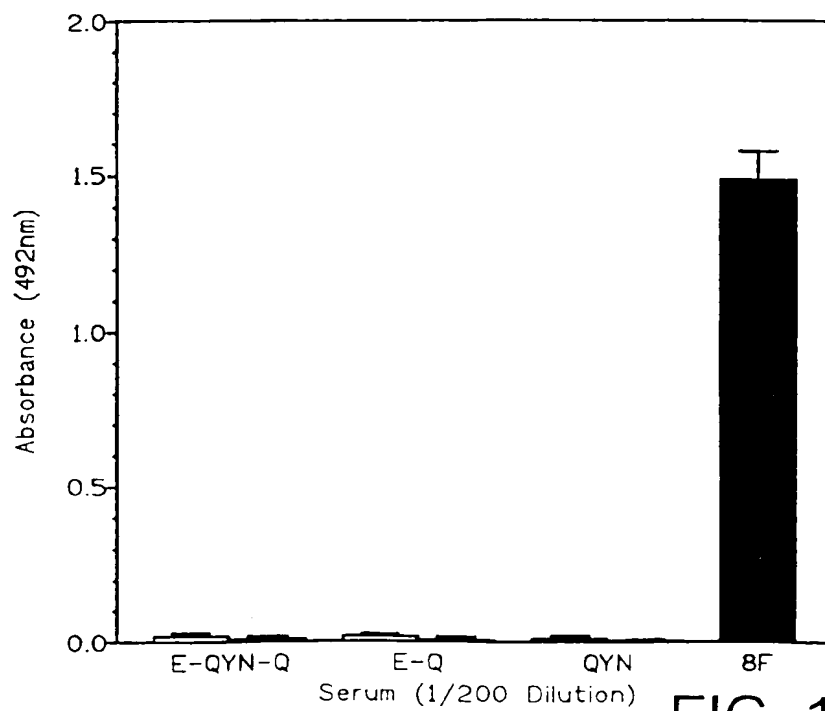
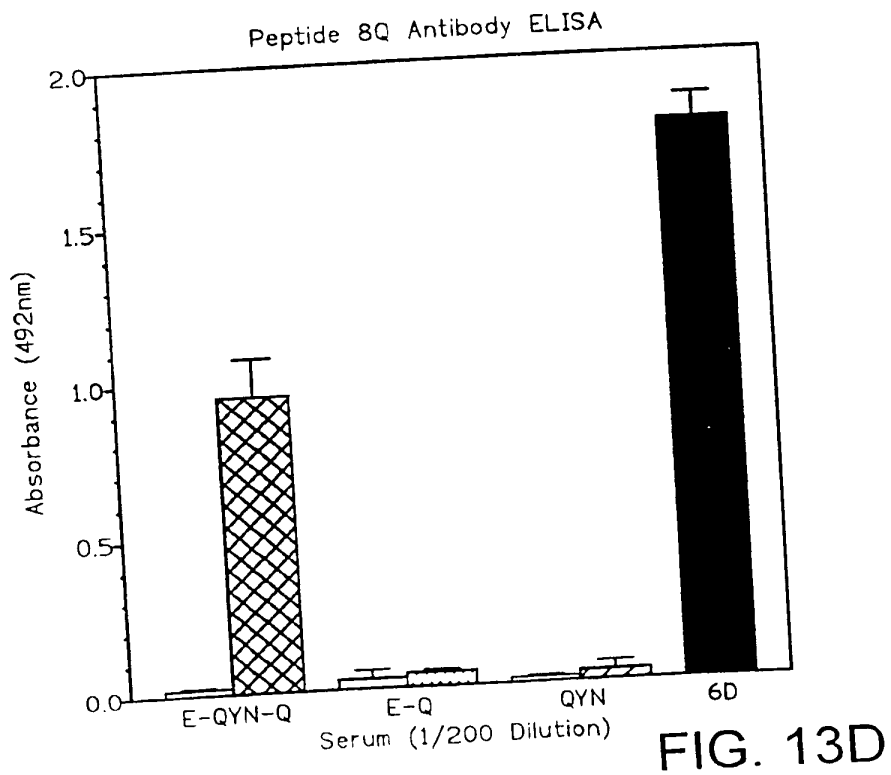
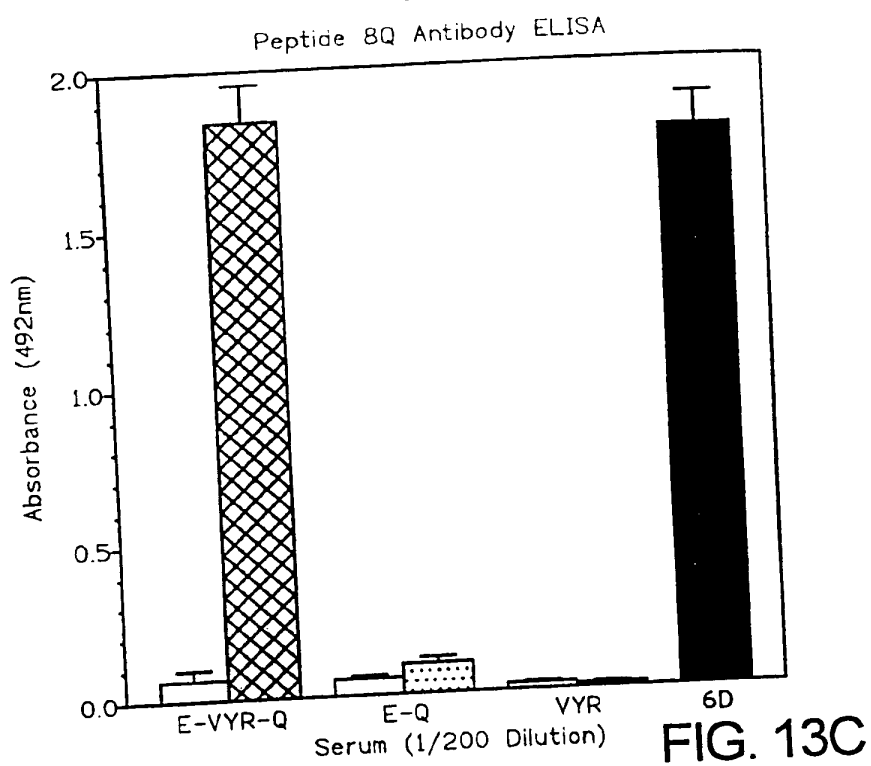


FIG. 13B

24/25



25/25

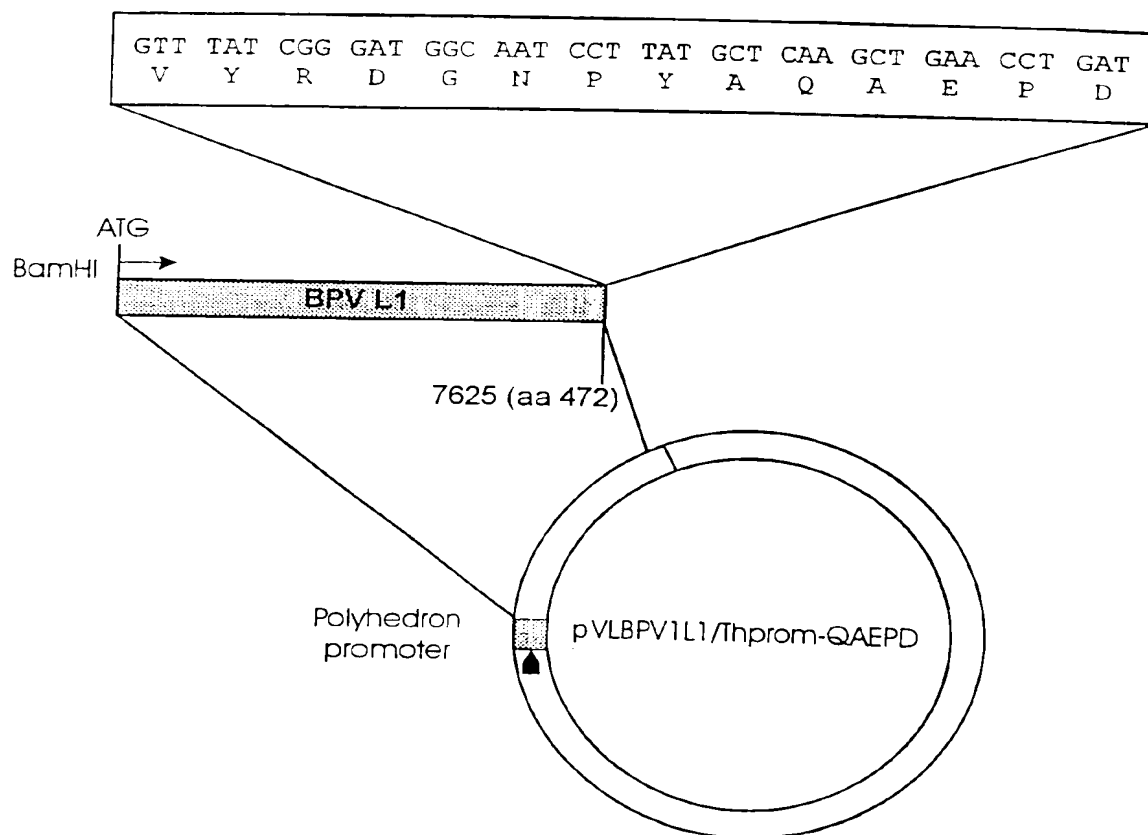


FIG. 14

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 97/00820

## A. CLASSIFICATION OF SUBJECT MATTER

Int Cl<sup>6</sup> C07K 4/02, 14/025, A61K 38/08, 38/10, 38/16, 39/12, 39/165, 39/385.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
STN Protein Subsequence Search (S VYRDGNPYA|QYNKPLCDLL/SQSP)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91/18294 (MEDSCAND AB) 28 November 1991 see in particular page 8, lines 27-32; page 38, line 22 and page 43, line 23.	21-24, 35-37
X	CANCER RESEARCH, Vol. 56, 1 September 1996, pages 3967-3974, T. Tsukui et al., 'Interleukin 2 Production in Vitro by Peripheral Lymphocytes in Response to Human Papillomavirus-derived Peptides: Correlation with Cervical Pathology'. see in particular page 3968, column 2, 'Peptides' and page 3969, Figure 1, peptides E6-G2, numbers 12, 19 and 20.	1-4, 15-17, 21-24, 35-37

☒ Further documents are listed in the  
continuation of Box C

☒ See patent family annex

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search  
7 January 1998

Date of mailing of the international search report  
19 JAN 1998

Name and mailing address of the ISA/AU  
AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION  
PO BOX 200  
WODEN ACT 2606  
AUSTRALIA Facsimile No.: (02) 6285 3929

Authorized officer

*G. A. Jenkins*  
GILLIAN JENKINS

Telephone No.: (02) 6283 2252

## INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/AU 97/00820

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH, Vol. 42, No. 5, 1 November 1993, pages 450-454, V. Krchnak et al., 'Aggregation of Resin-Bound Peptides During Solid-Phase Peptide Synthesis' see in particular page 452, Figure 1, panel A.	21-24
X	JOURNAL OF VIROLOGY, Vol. 68, No. 9, 1 September 1994, pages 5698-5705, S. A. Foster et al., 'The Ability of Human Papillomavirus E6 Proteins to Target p53 For Degradation in Vivo Correlates with Their Ability to Abrogate Actinomycin D Induced Growth Arrest'. see in particular page 5699, Figure 1, positions 53-61 and 91-100 and column 1, paragraph 2.	1-4, 15-17, 21-24, 35-37
X	VIROLOGY, Vol. 185, No. 2, 1991, pages 536-543, T. Kanda et al., 'Human Papillomavirus Type 16 E6 Proteins with Glycine Substitution for Cysteine in the Metal-Binding Motif.' see in particular page 537, Figure 1 and page 541, 'Discussion', paragraph 1.	1-4, 15-17 21-24, 35-37
X	VIROLOGY, Vol. 212, No. 2, 1995, pages 535-542, S. Nakagawa et al., 'Mutational Analysis of Human Papillomavirus Type 16 E6 Protein: Transforming Function for Human Cells and Degradation of p53 in Vitro.' see in particular page 536, Figure 1, HPV 16, positions 53-61 and 91-100 and page 537, 'Constructed HPV 16 E6 Mutants.'	1-4, 15-17 21-24, 35-37

# INTERNATIONAL SEARCH REPORT

## Information on patent family members

International Application No  
PCT/AU 97/00820

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	91/18294	AT	160020	AU	78890/91	AU	668499
		CA	2082658	CA	2040849	DE	69128152
		EP	594613	FI	925098	JP	6501542
		SE	9001705				

END OF ANNEX